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14. ABSTRACT
Xenotropic murine leukemia virus–related virus (XMRV) was recently discovered in human prostate cancers and is the first gammaretrovirus known to infect humans. While gammaretroviruses have well-characterized oncogenic effects in animals, they have not been shown to cause human cancers. We provide experimental evidence that XMRV is indeed a gammaretrovirus with protein composition and particle ultrastructure highly similar to Moloney murine leukemia virus (MoMLV). We analyzed 334 consecutive prostate resection specimens, using a qPCR assay and immunohistochemistry with an anti-XMRV specific antiserum. We found XMRV DNA in 6% and XMRV protein expression in 23% of prostate cancers. XMRV proteins were expressed in malignant epithelial cells, suggesting that retroviral infection may be directly linked to tumorigenesis. XMRV infection was associated with prostate cancer, especially higher-grade cancers. We found XMRV infection to be independent of a common polymorphism in the RNASEL gene, unlike previously reported. This result increases the population at risk for XMRV infection from only those homozygous for the RNASEL variant to all individuals. Our observations provide new evidence for an association of XMRV with malignant cells and with more aggressive tumors. XMRV displayed robust expression and infection in LNCaP prostate tumor cells. The transcriptional activity of the XMRV LTR was found to be higher than the MoMLV LTR in both LNCaP and WPMY-1 cells. The U3 promoter of XMRV and a glucocorticoid response element (GRE) in the U3 were required for transcriptional activity. Co-expression of the androgen receptor and stimulation with androgen stimulated XMRV-LTR dependent transcription in 293T cells and the GRE was required for this activity. Our data suggest that XMRV replicates more efficiently in certain prostate tumor cells in part due to the transcriptional environment in the prostate tumor cells.

15. SUBJECT TERMS
XMRV, retrovirus, malignant epithelium, qPCR, immunohistochemistry, long terminal repeat, LTR, transcription, luciferase

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INTRODUCTION

During the course of this award we have explored the possibility that XMRV, a newly-discovered retrovirus found to be associated with human prostate cancer, may be an initiating factor in prostate tumorigenesis. Recent work from the laboratory of Drs. Don Ganem and Joe DeRisi at UCSF led to the identification of a novel exogenous retrovirus, dubbed XMRV for Xenotropic Murine Leukemia Virus-Related Virus, present in a subset of human prostate tumor biopsies (13). XMRV sequences were originally recovered in a high proportion of tumors from patients homozygous for the susceptibility allele of the hereditary prostate cancer 1 (HPC1) locus, encoding a defective RNase L enzyme, and thus deficient in innate immunity to infectious agents. In the past two years, with the support of this grant, we have achieved several important goals. We have prepared replication-competent clones of the viral genome; analyzed its replication in many cell lines in laboratory cell cultures; determined the requirements on the viral genome for its highest transcription rates and gene expression; analyzed its sensitivity to the interferon response; demonstrated that the virus is found in malignant epithelial cells in prostate cancers, and more often in the more aggressive cancers; and documented the high-frequency presence of the virus in Leydig cells of the testes.

The origin of XMRV and its true prevalence in the human population have been highly controversial. Recent work has strongly suggested that the majority of the reported isolates in various laboratories were all derived from a single human prostatic cell line (22Rv1), one that was passed during its isolation through nude mice. This procedure has a long history of permitting the introduction of mouse xenotropic MLVs into the human cells, and this event almost certainly occurred during the derivation of 22Rv1. Sequence analysis of the virus in these cells indicates that it arose by recombination events between two endogenous proviruses in nude mice. Moreover, sequence analysis of the putatively distinct isolates in a number of laboratories indicates that many are not distinct but rather are contaminants derived from the 22Rv1 line. Studies of this XMRV strain have usefully addressed the properties of this virus, but most likely report on a virus of mouse origin. There is no reason to expect that the XMRV present in the 22Rv1 line is a cause of transformation in that tumor (it appeared in the line after the explant of the tumor), nor is there evidence that it is an authentic human pathogen. The virus is of fundamental interest – it replicates to extraordinarily high titers in human cells in culture, it infects primates efficiently, it replicates with interesting tissue tropisms, and it may yet be found to be a pathogen. But many of the early claims for its prevalence in the human population are unreliable, and attributable to the very high sensitivity of PCR methods used to detect low levels of viral DNA. Most clearly, the claims for association of XMRV with Chronic Fatigue Syndrome patients have been uniformly discredited.

The work from our laboratories and others has obtained evidence of XMRV-like viruses (or perhaps more properly xenotropic MLV-like viruses) in human tissues that are distinct from the 22Rv1 XMRV. In particular, our studies report PCR signals in tumor samples that are well-controlled with normal tissues, and in which samples are properly blinded to the investigators. We have employed immunological tools to screen tissue sections for virus gene expression and have detected viral antigens specific to tumor samples. Thus, it remains possible that xenotropic-like MLVs are circulating in

the human population and may be associated with prostate cancer. Going forward, proof of the presence of virus in a given sample will require PCR amplification and DNA sequence analysis to confirm that any new isolate is distinct from the XMRV in the 22Rv1 line. Even then, extraordinary measures must be taken to prevent contamination from other sources of mouse viruses and mouse DNA, including the reagents used in PCR protocols. New surveys that detect virus under these circumstances have the potential to ultimately determine the true prevalence of these viruses in humans and then to determine whether there is a link to any pathology.

BODY

Xenotropic Murine Leukemia Virus-Related Virus (XMRV) was discovered in prostate tumor cells that harbored a mutation of the HPC1 gene locus encoding RNase L. XMRV and other highly related polytropic murine leukemia virus (PMLV) sequences have been identified in Chronic Fatigue Syndrome (CFS) patients at a high incidence. Our work has focused on two areas: the characterization of the replication of the virus in various cell lines, with specific emphasis on the sensitivity of the virus to inhibition by the innate immune system; and the determination of the prevalence of the virus in prostate cancer patients and in the unaffected population, as well as the tissue distribution of the virus. We made significant contributions in each of these two areas.

Progress in Aim 1. Determinants of virus replication in cultured cell lines.

Numerous cell lines were tested for viral protein expression and for subsequent release of virus particles. 293T (human embryonic kidney), 2fTGH (human fibrosarcoma), HeLa (cervical carcinoma), and TE671 (rhabdomyosarcoma) produced XMRV virus particles but the titers were low. 293T cells and LNCaP cells were transfected with the provirus and monitored for the intracellular expression of XMRV gene products and the accumulation of virus particles in cell culture supernatants. Four and eight days post-transfection, viral proteins of both Moloney MLV (MoMLV) and XMRV were detected in the media. 293T cell supernatants contained a very small amount of XMRV viral capsid compared to the MoMLV control. In contrast, XMRV virus protein accumulation was much higher and the same as MoMLV virus particles in LNCaP cells. Over time, XMRV protein accumulated to higher degree than MoMLV consistent with the fact that MLVs cannot spread infection in human cells. Significantly, more XMRV protein was observed in LNCaP cells than in 293T at both time points. Cell lysates prepared at day eight of the experiment clearly show unprocessed Gag protein and the capsid (CA) cleavage product of XMRV accumulating to high levels in prostate LNCaP cells but not 293T cells. Functional RT activity was also detected in the media of LNCaP cells suggesting that significant numbers of viral particles were being produced only from LNCaP cells.

Next it was determined whether XMRV virus particles produced from LNCaP cells are infectious. Non-infected 293T and LNCaP cells were exposed to LNCaP cell culture supernatants to allow XMRV to adsorb. After replacing the media, RT activity of the cell culture supernatants was measured on consecutive days to monitor release of viral particles. RT activity was detected in the LNCaP but not 293T cell supernatant

indicating that XMRV is conducting a spreading infection more efficiently in prostate cells.

The ability of XMRV to perform a spreading infection in three additional cell lines was tested next. XMRV supernatants from LNCaP cells were applied to HeLa (human cervical carcinoma), TE671 (human rhabdomyosarcoma), 2fTGH cells (human fibrosarcoma), and monitored for RT release for three days post-infection. Only prostate LNCaP cells efficiently supported a spreading infection, suggesting cell-type specificity for XMRV replication.

Multiple hypotheses could explain why XMRV replicates and spreads better in human prostate cells compared to cells of other tissues. First, it is possible that prostate cells may express the cellular receptor while other cell types do not. Expression of XPR1 in prostate cells may confer an entry advantage over other cell types that do not express XPR1, or express the receptor at very low levels. Second, RNase L is thought to be a XMRV restriction factor since most of XMRV-positive tumor samples contained a slightly inactivating mutation in the RNase L gene, HPC1. Some prostate cells lines may indeed have this mutation. Although both theories merit further research, we decided to focus on a third mechanism, namely that prostate cells provide a much more favorable transcriptional environment than other cell types. This would potentially allow for increased viral protein expression and viral release cell culture supernatants.

To test this hypothesis, the 5′ LTR of the integrated proviruses from both MoMLV and XMRV up to the Gag expression start site were fused with the luciferase gene. This expression vector can then test the transcriptional output of both MoMLV and XMRV LTRs once they were transfected into different cell types. We also generated a series of 5′ and 3′ deletions to assign specificity to the transcriptional output. The expression vectors were initially transfected into LNCaP cells to test their transcriptional activity. Interestingly the XMRV LTR had much higher (approximately six-fold) transcriptional activity than MoMLV in LNCaP prostate cells. This activity decreased by half when the R, U5, and the untranslated regions were deleted individually. However, any deletion of within the 5′ region of U3 dramatically inhibited the transcriptional activity of XMRV. We also transfected the LTR-luciferase fusion reporters into 293T, LNCaP and YPMY-1 cells and quantified their transcriptional output. Significantly, MoMLV activity was higher than XMRV in 293T cells but was lower than XMRV in both LNCaP and YPMY-1 cells. This suggests that XMRV viral particle production depends on the transcriptional environment provided by prostate cells.

These data suggest that the U3 region of the XMRV LTR plays a critical role in transcriptional activation in LNCaP cells. To determine whether the XMRV U3 is necessary and sufficient for transcription in LNCaP cells, a chimeric fusion construct between MoMLV and XMRV was created and fused to luciferase. This reporter has the XMRV U3 fused to the R, U5 and untranslated region of MoMLV. This, along with MoMLV and XMRV controls, were then transfected into 293T, YMPY-1, or LNCaP cells and analyzed for luciferase activity. As expected, the XMRV transcriptional activity was higher in prostate cells compared to 293T cells. Importantly, the reporter that only contained the XMRV U3 was phenotypically similar to XMRV indicating that the U3 is responsible for the observed differences between non-prostate and prostate cells.

Although the pathogenicity of XMRV is still unknown, we felt that a better understanding of interactions between XMRV and host innate immunity was warranted. We found that XMRV viral spreading is blocked by Interferon (IFN), an innate immunity antiviral cytokine. To show that XMRV is indeed restricted by IFN, we utilized a human fibrosarcoma cell line, 2fTGH, which is competent in IFN signaling. We also used derivatives of the same cell line that harbored a somatic gene mutation resulting in the lack of either STAT1 (U3A cells) or STAT2 (U6A cells) expression. 2fTGH cells are capable of a sustained IFN response as indicated by the increased endogenous expression of STAT1 and STAT2, both being IFN-inducible. However U3A cells, which lack STAT1, do not show an increase in steady-state levels of STAT2. 2fTGH and U3A cells were pre-treated with IFN for twenty-four hours, infected with XMRV virions, and analyzed for viral release into the cell culture media. Both wild-type and STAT1 deficient cell lines supported XMRV viral spreading, as indicated by accumulation XMRV capsid (CA) in the culture media. However, IFN reduced XMRV replication by three days and completely inhibited spread by six days in wild-type 2fTGH cells, consistent with other studies demonstrating that XMRV is sensitive to IFN. Importantly, in the absence of STAT1 or STAT2 IFN did not restrict XMRV spreading as indicated by accumulated XMRV CA protein. Next, wild-type cells, STAT1 deficient or STAT2 deficient cells were infected with a single round Moloney murine leukemia virus (MoMLV) expressing firefly luciferase pseudotyped with XMRV Envelope (Env). Pre-treating 2fTGH cells with IFN reduced infection of the pseudotyped XMRV reporter virus, but not in cells lacking either STAT1 or STAT2. Together these data indicate that STAT1 and STAT2-mediated IFN signaling can restrict XMRV viral spreading. Moreover, this inhibition may occur at an early step in the viral life cycle or before budding and release of the virion.

As retroviral restriction factors, APOBEC3G and Tetherin have the potential to inhibit replication of XMRV. To demonstrate this restriction, we overexpressed both APOBEC3G and Tetherin to block early and late steps of the viral life cycle, respectively. A single-round Moloney MLV reporter virus expressing luciferase and pseudotyped with XMRV Env was capable of infecting 2fTGH cells. However, increased co-expression of exogenous APOBEC3G reduced luciferase activity in a dose-dependent manner, indicating APOBEC3G can block early time points of infection. Next, we analyzed the ability of Tetherin to block release of XMRV in 293T cells. Transfection of 293T cells with the XMRV provirus and analysis of the culture media pellets revealed an accumulation of XMRV CA, indicating release of the virions into the media. Co-expression of endogenous Tetherin, however, completely blocked release of XMRV. The vpu accessory protein of HIV, which can induce the degradation of Tetherin, can dose-dependently relieve the restriction by Tetherin as indicated by accumulation of CA in the culture media. Together these data confirm that APOBEC3G and Tetherin are potent inhibitors of XMRV.

We next tested whether endogenous IFN-induced APOBEC3G or Tetherin in 2fTGH cells are capable of blocking XMRV in the context of an IFN response. Small hairpin RNAs (shRNA) targeting APOBEC3G and Tetherin for RNAi were stably introduced into 2fTGH cells and resulted in the reduction of mRNA to greater than 90%

when compared to a scrambled hairpin control as revealed by quantitative real-time PCR. Moreover, while IFN induced Tetherin mRNA more than fifty-fold compared to control or seven-fold for APOBEC3G, the shRNAs silenced APOBEC3G and Tetherin gene induction in response to IFN. However, despite robust APOBEC3G and Tetherin gene silencing, IFN was still able to restrict XMRV infection and replication.

The data indicate that although IFN can inhibit XMRV, RNAi-mediated reduction of APOBEC3G or Tetherin does not relieve IFN-mediated restriction. Other branches of the IFN pathways must be the major players in restriction of XMRV. To determine the time of the block, wild-type cells and STAT1 deficient U3A cells were pre-treated with IFN and infected with XMRV. Genomic and small molecular weight DNA was harvested twenty four hours post infection (hpi) and subjected to quantitative real-time PCR for amplification of the XMRV minus strong stop sequence (XMSS), a product of retroviral reverse transcription. The XMSS was detected upon infection with media containing XMRV virus particles but was reduced five-fold by pre-treating with IFN. This inhibition did not occur in U3A cells again indicating that STAT1 is required for IFN inhibition of XMRV. This data suggests that IFN is blocking XMRV replication at or before retroviral reverse transcription.

Progress in Aim 2. Transgenic models for XMRV pathogenesis.

We elected to abandon efforts toward the generation of a transgenic receptor-based mouse model for XMRV disease in light of two important findings in the field: First, we now know that wild mice and rat lines and whole animals, in contrast to inbred laboratory mouse lines and animals, express a receptor that renders them susceptible to XMRV. Thus, rat provides a ready model for virus-induced prostate disease. Second, we have learned that a transgene of the XMRV provirus in mice, although virus spread does not occur, nevertheless can induce a prostate inflammation similar to the human condition (L. Ratner, Wash. U., personal communication). These systems thus provide adequate small animal models for future studies of XMRV replication and pathogenesis.

Progress in Aim 3. Examination of human prostate biopsies for XMRV expression, and determination of tissue distribution of virus-infected cells.

We obtained large numbers of de-identified human prostate cancers, in the form of frozen and paraffin-embedded tissues, of different grades, stages and histologic subtypes. We have characterized the presence and localization of XMRV within these cancers using two distinct readouts and methodologies. We have probed histological sections of human prostate cancers and also benign prostates, a total of 334 consecutive prostrate resections, for viral protein expression and tested for presence of XMRV proviral DNA using quantitative real time PCR. We found XMRV DNA or protein in over one-fourth of prostate cancers. XMRV proteins were expressed primarily in malignant epithelial cells. XMRV infection was associated with prostate cancer, especially higher-grade cancers. We found XMRV infection to be independent of a common polymorphism in the RNASEL gene, unlike results previously reported (13). This finding increases the population at risk for XMRV infection from only those

homozygous for the RNASEL variant to all individuals. The findings have been summarized in an important publication in the field (11 and appendix).

The human prostate is composed primarily of acinar or ductal epithelial cells, which serve the secretory function of the gland. Almost all cases of human prostate cancer are the result of malignant proliferation of these epithelial cells. In addition to the epithelial cells, the prostate also contains stromal cells, primarily fibroblasts, with a few macrophages, lymphocytes and an occasional granulocyte. To render a pathologic diagnosis of cancer, the entire prostate gland is routinely sampled, resulting in an average of 20-30 tissue blocks that are eventually banked in the tissue repository. Prostate cancer usually follows a focal pattern, with malignant cells seen in only a minority of the sampled tissue blocks. Within each block, the extent of cancer also varies greatly. We examined sections stained with hematoxylin and eosin dyes that were prepared from each block for routine diagnostic purposes. For each case, we selected one or two tissue blocks that contained the highest proportion of malignant epithelial cells for IHC analysis. For every case that showed any staining with IHC, we also tested adjacent sections with control pre-immune serum.

We applied our optimized IHC protocol to prostate tissue sections. In brief, XMRV proteins were expressed in prostatic tissues from 23% with prostate cancer and in 4% without prostate cancer. Interestingly, and in contrast to previous reports, staining was predominantly observed in malignant epithelial cells. Of the IHC-positive cases with prostate cancer, expression of XMRV protein was observed in epithelial cells in 85% of cases, in both epithelial and stromal cells in 7.5% of cases, and exclusively in stromal cells in another 7.5% cases.

Epithelial cells expressing XMRV protein were usually seen clustered in an acinus or in a few acini adjacent to each other. The proportion of cells expressing XMRV protein in a given tissue section varied widely from case to case. However, in all cases the positively staining cells represented the minority of cells in the section. Staining intensity also varied between cases ranging from intense staining of the entire cytoplasm to more discrete staining in which case the granular nature of the staining could be more readily appreciated. The vast majority of IHC-positive epithelial cells showed the same granular staining pattern of the entire cytoplasm described above. In a small number of cases we observed epithelial staining of only a circumscribed portion of the cytoplasm. Rare scattered XMRV-expressing stromal cells were seen in proximity to malignant acini or in lymphocytic infiltrates adjacent to malignant acini. Over all, the number of stromal cells expressing XMRV protein was much smaller than the number of IHC-positive epithelial cells (data not shown).

In summary, we observed expression of XMRV protein in 23% of prostate cancer cases and in only 4% of control cases. We identified XMRV protein most frequently in clusters of epithelial cells that were part of the cancer. Rare stromal cells expressing XMRV protein showed a staining pattern similar to that reported previously. The odds for XMRV being detected in prostate tissues from men with prostate cancer was more than 5 times higher than those for XMRV being present in tissue samples from men without prostate cancer (OR = 5.7, $p \ll 0.001$). We therefore identified a strong correlation between the presence of XMRV DNA and prostate cancer in our study population, using qPCR and XMRV-specific IHC assays for detection of XMRV DNA

and protein, respectively. An analysis of tumor samples to show the relationship of the virally infected cells to tumor morphology, grade and stage is in progress.

We extended our analysis to tissues obtained at autopsy. We have looked for XMRV in a large panel of organs from 72 autopsies performed on men with and without a diagnosis of prostate cancer. This is the largest study we are aware of that attempts to examine tropism of XMRV in human tissues. Consistent with previous results, XMRV proteins were detected in 2 of 6 (33%) prostate cancers but in none of the 64 benign prostate samples. XMRV was undetectable in most non-prostatic tissues examined but we did detect XMRV in the testes of 86% of men with prostate cancer, regardless of whether it was detected in the corresponding prostates. Intriguingly, XMRV was also detected in the testes of 54% of men without a diagnosis of prostate cancer. Within the testes, XMRV staining was localized to the androgen-secreting interstitial cells of Leydig - not in germ cells. We found that Leydig cells do not express APOBEC3G, a retroviral restriction factor with anti-XMRV activity. Our results suggest that testicular Leydig cells could serve as a reservoir for XMRV, with their lack of APOBEC3G resulting in release of replication-competent virus. Our data also suggest that XMRV prevalence might be much greater than previously recognized.

The work done in our laboratories has been performed under strict conditions to prevent contamination by 22Rv1-derived virus, or by mouse DNA in the reagents. Further, the samples were blinded, and control samples were always included. Nevertheless, these studies can always be criticized as potentially attributable to contamination. The PCR products amplified from fixed tissues are too short to allow for DNA sequence-based differentiation of the isolates from 22Rv1 DNA. The immunocytochemistry assays are not subject to PCR artifacts, but the antigens detected by the polyclonal antisera may be nonviral in origin, attributed to cross-reactivity with XMRV proteins, or expressed from endogenous proviral (HERV) genomes. Our data remains probably the most suggestive that xenotropic MLV-like sequences are found in prostate cancer samples, but only further work will determine the true prevalence of these sequences in the human population, and ultimately, whether or not they are responsible for prostate cancer.

KEY RESEARCH ACCOMPLISHMENTS

We made significant progress in the work outlined in Aims 1 and 3 of our original proposal. With respect to Aim 1, we have propagated virus in cultured cells, and determined the tissue range of the virus, being remarkably tropic for prostate cell lines. We provide strong evidence that a major basis for this tropism is the steroid-responsiveness of the viral promoter encoded in the U3 region of the LTR. We further have shown that the virus is highly sensitive to interferon, and that the restriction of virus requires the STAT signaling molecules. Further, we show that although the interferon induced genes APOBEC and Tetherin do inhibit virus, nevertheless at least part of the restriction must be attributed to the activity of novel interferon-responsive gene(s) that act before or during reverse transcription.

Recent findings have made efforts to develop a transgenic animal model, our original Aim 2, unnecessary. It has been discovered that a strain of wild mice, *Mus*

pahari, possesses functional XPR1 receptors, is susceptible to XMRV infection, and mounts an anti-XMRV immune response (14). It remains to be seen if the animals will develop prostate cancer.

With respect to Aim 3, we made very strong progress. We analyzed 334 consecutive prostate resection specimens, using a quantitative PCR assay and immunohistochemistry (IHC) with an anti-XMRV specific antiserum. We found XMRV DNA in 6% and XMRV protein expression in 23% of prostate cancers. XMRV proteins were expressed primarily in malignant epithelial cells, suggesting that retroviral infection may be directly linked to tumorigenesis. XMRV infection was associated with prostate cancer, especially higher-grade cancers. We found XMRV infection to be independent of a common polymorphism in the RNASEL gene, unlike results previously reported. This finding increases the population at risk for XMRV infection from only those homozygous for the RNASEL variant to all individuals. Our observations provide evidence for an association of XMRV with malignant cells and with more aggressive tumors (11).

REPORTABLE OUTCOMES

1. We constructed an infectious clone of XMRV, which produced infectious virions when transfected into cells.
2. When analyzed by transmission electron microscopy, XMRV virions produced in cell culture resembled other type-C retroviruses in morphology.
3. The infectious clone of XMRV replicated in human cell lines such as 293Ts, but was especially efficient at replication in prostate cancer cell lines such as LNCaP cells.
4. Transcriptional activity of XMRV was higher than MoMLV in LNCaP prostate cells and may be a significant factor in determining cell-type specificity for XMRV protein expression and viral particle accumulation.
5. Deletion analysis suggested that the U3 region of the XMRV LTR plays a critical role in transcriptional activation in LNCaP cells.
6. We designed a sensitive and specific quantitative PCR assay to detect XMRV DNA from frozen or formalin-fixed, paraffin-embedded prostate tumors.
7. We generated antisera that are highly specific for XMRV.
8. We designed an immunohistochemistry protocol that specifically detects XMRV protein expression in prostate cancers.
9. XMRV proteins are expressed mostly in malignant epithelial cells. Benign cells do not express XMRV proteins and very rarely do stromal cells express XMRV proteins.

Two reports of our results have been published (see Appendix).

Two more publications are in preparation: one documenting the IFN-susceptibility of XMRV, and one reporting the prevalence of XMRV in Leydig cells both in healthy and affected humans.

CONCLUSIONS

We made significant progress in the characterization of the novel XMRV virus, documenting its tissue tropism and interferon-sensitivity. We also documented the prevalence of the virus in human populations, both in prostate and most recently in Leydig cells of the testes.

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APPENDIX

Publications arising from this research

XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors.
Schlaberg R, Choe DJ, Brown KR, Thaker HM, Singh IR.
Proc Natl Acad Sci U S A. 2009 Sep 22;106(38):16351-6.

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Rodriguez JJ, Goff SP.
J Virol. 2010 Mar;84(5):2556-62.

XMRV is present in malignant prostatic epithelium and is associated with prostate cancer, especially high-grade tumors

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Xenotropic murine leukemia virus–related virus (XMRV) was recently discovered in human prostate cancers and is the first gammaretrovirus known to infect humans. While gammaretroviruses have well-characterized oncogenic effects in animals, they have not been shown to cause human cancers. We provide experimental evidence that XMRV is indeed a gammaretrovirus with protein composition and particle ultrastructure highly similar to Moloney murine leukemia virus (MoMLV), another gammaretrovirus. We analyzed 334 consecutive prostate resection specimens, using a quantitative PCR assay and immunohistochemistry (IHC) with an anti-XMRV specific antiserum. We found XMRV DNA in 6% and XMRV protein expression in 23% of prostate cancers. XMRV proteins were expressed primarily in malignant epithelial cells, suggesting that retroviral infection may be directly linked to tumorigenesis. XMRV infection was associated with prostate cancer, especially higher-grade cancers. We found XMRV infection to be independent of a common polymorphism in the *RNASEL* gene, unlike results previously reported. This finding increases the population at risk for XMRV infection from only those homozygous for the *RNASEL* variant to all individuals. Our observations provide evidence for an association of XMRV with malignant cells and with more aggressive tumors.

Gleason | immunohistochemistry | retrovirus | RNaseL | xenotropic

Prostate cancer is the most common form of nonskin cancer in U.S. men (1). The lifetime risk for developing prostate cancer is ≈1 in 6 (2) in the United States, and globally, 3% of men die of prostate cancer (3). Morbidity and mortality from prostate cancer are likely to grow further, given increasing longevity. Epidemiologic studies indicate that infection and inflammation may play a role in the development of prostate cancer (4, 5). A search for viral nucleic acids in prostate cancers led to the identification of xenotropic murine leukemia virus–related virus (XMRV) in ≈10% of samples tested (6). Because only malignant tissues were analyzed in the initial report, an association of XMRV with prostate cancer could not be addressed. Our analysis of 233 cases of prostate cancers and 101 benign controls showed an association of XMRV infection with prostate cancer, especially with more aggressive tumors. XMRV proteins were almost exclusively expressed in malignant epithelial cells. Only rarely did we find XMRV proteins in benign stromal cells, in contrast to a previous report (6).

XMRV was originally discovered in patients with a reduced activity variant of the *RNASEL* gene, and a strong correlation between this variant (R462Q) and the presence of XMRV was reported: 89% of XMRV-positive cases and only 16% of XMRV-negative cases were homozygous (QQ) for this variant in a total of 86 cases (6). Our study of 334 cases allowed us to establish the independence of XMRV infection and the R462Q variant. This finding moves the population at risk for XMRV infection from a small, genetically predisposed fraction homozygous for the R462Q *RNASEL* variant to all men. Sequence comparisons have classified XMRV as a gammaretrovirus with a high similarity to murine leukemia viruses. We present experimental evidence that XMRV is indeed a gammaretrovirus. Gammaretroviruses cause leukemias and sarcomas in multiple rodent, feline, and primate species but have not yet been shown to cause cancers in humans. Taken together, our findings provide evidence consistent with a direct oncogenic effect of this recently discovered retrovirus. If established, a direct role for XMRV in prostate cancer tumorigenesis would open up opportunities to develop new diagnostic markers as well as new methods to prevent and treat this cancer with antiretroviral therapies or vaccines.

Results

A Molecular Clone of XMRV Infects Human Prostate Cells. We constructed pXMRV1, a full-length XMRV molecular clone, using 2 overlapping clones from patient isolate VP62 (6) [gift of Don Ganem, University of California, San Francisco (UCSF)]. pXMRV1 was transfected into 293T cells. Reverse transcriptase (RT) activity was detected in the supernatant within 1–2 days of transfection (Fig. 1*A*), indicating the release of viral particles. These were inoculated onto naive 293T cells and LNCaP cells, a human prostate cancer cell line (American Type Culture Collection CRL-1740). Viral release from infected LNCaP cells was first seen on day 7 postinoculation and peaked at day 12. No particles were released from similarly inoculated 293T cells up to day 14. pXMRV1 is therefore an infectious molecular clone, and XMRV replicates efficiently in human prostate cells.

XMRV Particles Have Type-C Retrovirus Morphology. Particles released from XMRV-infected cells closely resembled those of a gammaretrovirus, Moloney murine leukemia virus (MoMLV), in size and morphology (Fig. 1 *B–E*). XMRV particles had an average diameter of 137 nm (SD = 9 nm), a spherical to somewhat pleomorphic shape, and characteristic lipid envelopes. The majority of particles contained an electron-dense, polygonal core with an irregular outline (average diameter 83 nm, SD = 8 nm), resembling mature type-C retroviral cores (Fig. 1*C*). Cores defined as "immature," i.e., spherical with an electron-lucent center, were also seen (Fig. 1*D*). A "railroad track," a term used to describe immature MoMLV cores (7), and formed by the radial alignment of the N- and C-terminal halves of the CA protein, was also seen in immature XMRV cores (Fig. 1*D*, arrowhead). These striking ultrastructural similarities between XMRV and MoMLV (Fig. 1*E*) suggest that the 2 viruses are assembled in a very similar manner.

XMRV Proteins, Except for Env, Closely Resemble Those of MoMLV. We identified XMRV proteins and defined their molecular weights by

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The authors declare no conflict of interest.

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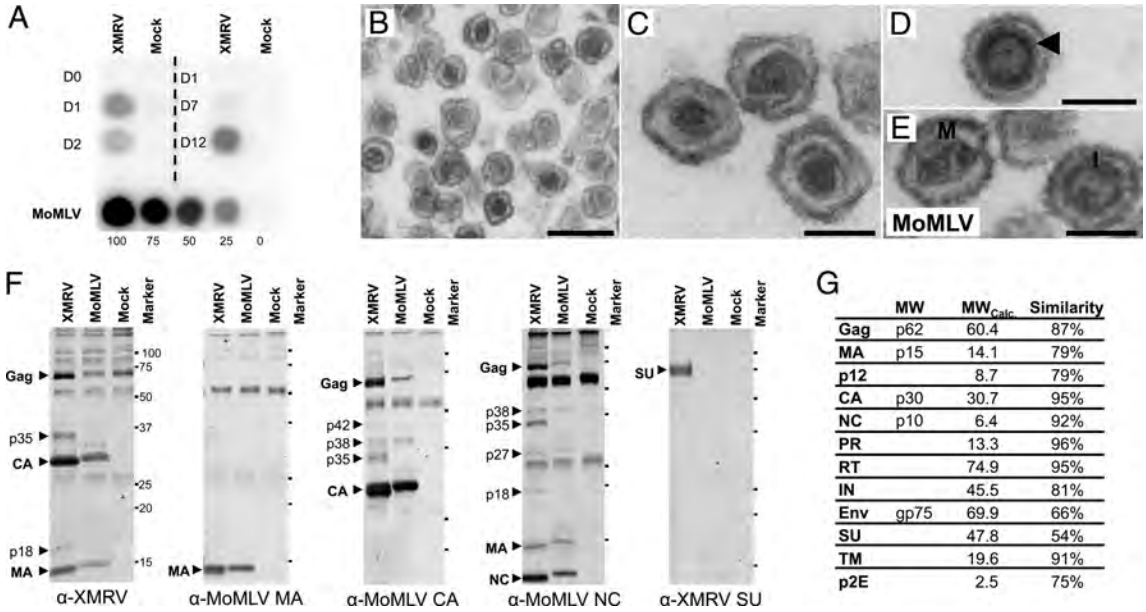

	MW	$MW_{Calc.}$	Similarity
Gag	p62	60.4	87%
MA	p15	14.1	79%
p12		8.7	79%
CA	p30	30.7	95%
NC	p10	6.4	92%
PR		13.3	96%
RT		74.9	95%
IN		45.5	81%
Env	gp75	69.9	66%
SU		47.8	54%
TM		19.6	91%
p2E		2.5	75%

Fig. 1. The XMRV molecular clone produces infectious particles with morphology and composition similar to MoMLV. (*A*) Viral release from cells transfected or inoculated with pXMRV1 or XMRV, respectively. (*Left*) Reverse transcriptase (RT) activity in culture supernatants from cells transfected with pXMRV1 or control EGFP plasmid. (*Right*) RT activity from LNCaP cells inoculated with XMRV. (*Lower*) RT activity from NIH 3T3 cells chronically infected with MoMLV shown for comparison. (*B–E*) Transmission electron microscopy of XMRV particles (*B*), mature XMRV cores (*C*), immature XMRV core, with ''railroad track'' marked by arrowhead (*D*), and MoMLV particles with mature (''M'') and immature (''I'') cores (*E*). (*F*) Western blot analysis of lysed XMRV and MoMLV virions, using antisera to XMRV whole virus, MoMLV-CA, MoMLV-MA, MoMLV-NC, and XMRV-Env SU. Comparison of blots allows identification of intermediates of Gag proteolysis, e.g., p27 (MA-p12), p42 (p12-CA), and p38 (CA-NC). (*G*) Molecular weights of XMRV proteins as calculated by Western blot analysis and by sequence prediction and similarity between XMRV and MoMLV proteins. [Scale bars: 250 nm (*B*) and 100 nm (*C–E*).]

comparing Western blots of lysed XMRV and MoMLV virions probed with antisera specific to XMRV or to MoMLV Gag proteins (Fig. 1 *F* and *G*). In accordance with their high (≈90%) sequence similarities, the molecular weights of XMRV and MoMLV Gag proteins were found to be very similar. We identified a 75-kDa band as the surface unit (SU) of the envelope (Env) protein, using rabbit antiserum specific to XMRV-Env SU. This antiserum did not react with the MoMLV-SU, consistent with the lower sequence similarity (54%) of the corresponding Env proteins and the general tendency of Env proteins to show greater evolutionary divergence, as compared to Gag or Pol proteins.

XMRV Proviral DNA Is Detected in 6% of Human Prostate Cancers; Viral Loads of XMRV Are Low. Our quantitative (q)PCR was designed to efficiently amplify XMRV proviral DNA from formalin-fixed, paraffin-embedded (FFPE) tissues. Primers and probes were chosen in a region of the integrase gene that is 100% conserved between all 3 published XMRV isolates and yet shares at most 80% similarity with the most closely related murine retroviral sequences (Fig. S1*A*). A common forward primer was used with 2 different reverse primers to allow for sequence differences in clinical isolates. Our qPCR was specific for XMRV sequences and did not amplify murine or human endogenous retroviruses; no amplification products were seen when using C57BL/6 mouse genomic DNA or human placental DNA as template. We tested the sensitivity of our qPCR assay in 2 ways. First, in the presence of excess human placental DNA, we could consistently detect 50 copies of the XMRV proviral clone and 5 copies 50% of the time (Fig. S1*B*). Second, because formalin fixation and embedding in paraffin compromise DNA quality, we also used fixed templates to test sensitivity. When DNA from FFPE human prostate tissue sections was spiked with known dilutions of DNA from fixed and embedded XMRV-infected, cultured cells, we consistently detected 1–2 infected cells per qPCR sample (Fig. S1*C*). We developed a second qPCR targeting the single-copy gene–vesicle-associated membrane protein 2 (VAMP2) to test for DNA integrity and amplification inhibitors [details in supporting information (*SI*) *Text*].

To estimate the prevalence of XMRV in men with and without prostate cancer, we analyzed 233 consecutively accessioned prostate cancers and 101 cases of transurethral resection of the prostate (TURP) as benign controls (Fig. S1*D*). We detected XMRV DNA in 14 (6.2%) cases of prostate cancer and in 2 (2.0%) controls. We determined XMRV proviral loads in these tissues. Using XMRV plasmid DNA as a standard, we estimated that qPCR-positive prostate cancers contained 1–10 copies of XMRV DNA per 660 diploid cells (see *Materials and Methods* and Fig. S1*E*). Because the number of tumor cells in any given section varies widely between tumors and even between different areas in the same tumor, it is impossible to estimate how many copies of XMRV DNA are present in each *tumor* cell. Using FFPE XMRV-infected cells as standards, we calculated that each 10-μm section from a prostate cancer contained the same amount of proviral DNA as 6–7 XMRV-infected cultured cells.

XMRV Protein Is Expressed in 23% of Prostate Cancers and Is Predominantly Seen in Malignant Epithelium. We developed XMRV-specific antisera and used them for immunohistochemistry (IHC). We first used XMRV-infected and uninfected cells that were mixed at different ratios and fixed in formalin and embedded in paraffin to mimic prostate tissue sections. We saw granular cytoplasmic staining in cells in proportion to the percentage of infected cells in the corresponding mixtures (Fig. 2 *A–C*). No staining was seen in uninfected cells or with preimmune serum (Fig. S2 *A* and *B*), confirming the specificity of our assay. We next performed IHC on prostate samples from XMRV qPCR-positive cases. We saw the same cytoplasmic granular pattern in tissues as in infected cultured cells (Fig. 2 *D* and *E*). Antiserum from a second rabbit resulted in identical staining. No staining was seen with preimmune serum (Fig. 2*F*).

We tested tissue sections from all 334 cases of prostate cancer and

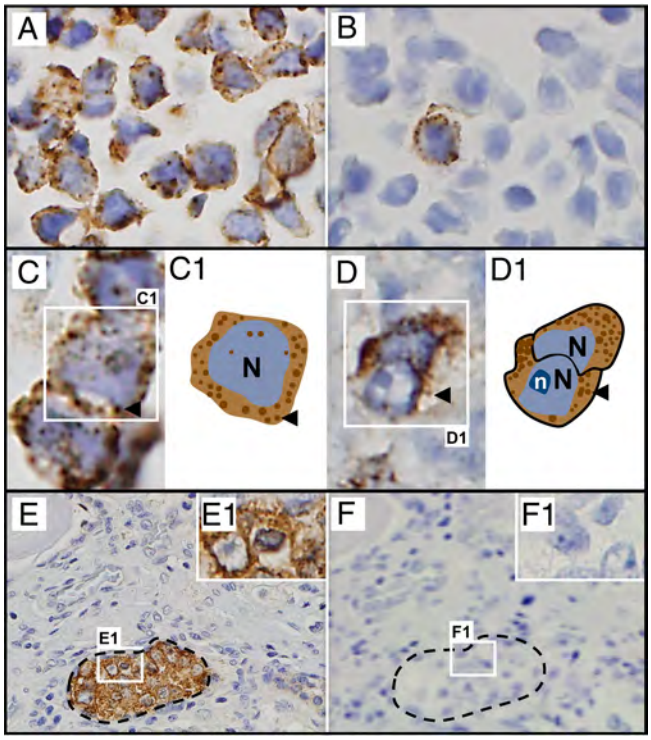

Fig. 2. XMRV proteins detected in infected cultured cells and in prostate cancer tissue by IHC, using anti-XMRV antiserum. Counterstaining with hematoxylin reveals blue nuclei. (*A* and *B*) XMRV-infected cells: 100% infected (*A*) and 1% infected (*B*). (*C*) Cultured infected cells at higher magnification show cytoplasmic granular staining, represented diagrammatically in *C1* (arrowhead, granules). (*D–F*) Human prostate cancers with clusters of malignant epithelial cells (*E*), with *Inset* at higher magnification (*E1*). Granular staining pattern seen at higher magnification. (*F* and *F1*) Adjacent section stained with preimmune serum from the same rabbit. N, nucleus; n, nucleolus.

controls with benign prostatic hyperplasia. We found XMRV protein expression in 54 (23%) cases with prostate cancer and in 4 (4%) controls (Fig. 4*A*). In contrast to a previous report (6) that found XMRV-specific staining only in nonmalignant stromal cells, we observed XMRV-specific staining predominantly in malignant prostatic epithelial cells. XMRV proteins were expressed in epithelial cells in 46 tumors (85%), in both epithelial and stromal cells in 4 tumors (7.5%), and exclusively in stromal cells in another 4 tumors (7.5%). Of the 4 controls, XMRV expression was seen in epithelial cells in 3 and in both epithelial and stromal cells in 1 case. Epithelial cells expressing XMRV protein usually belonged to a single acinus or to a few adjacent acini. The proportion of cells expressing XMRV protein in a given tissue section varied widely (Fig. 3 *A–G*) but positive cells always represented a minority of cells on the slide. The vast majority of IHC-positive epithelial cells showed the same granular staining pattern of the entire cytoplasm that was seen in cultured cells (Fig. 3 *A–F*). However, the staining intensity and the subcellular pattern varied between cases, ranging from intense staining of the entire cytoplasm (Fig. 3*E*) to more discrete granular staining (Fig. 3 *C* and *D*), with some unusual staining patterns (Fig. 3*G*). In summary, XMRV proteins were expressed in 23% of prostate cancers and 4% of controls. Protein expression was seen in clusters of malignant epithelial cells and very rarely in stromal cells (Fig. 3 *H* and *I*).

Presence of XMRV Correlates with Prostate Cancer and Higher Tumor Grade. We tested for a correlation of XMRV positivity (by qPCR or IHC) with the presence, grade, and stage of prostate cancer. XMRV positivity was 5-fold higher in cancer than in benign controls (odds ratio = 5.7, $P < 0.0001$, Fig. 4*A*). We also tested for a correlation between XMRV positivity and tumor grade as measured by the Gleason score. We saw a correlation between XMRV positivity and higher-grade cancers (Fig. 4*B*). Of the 233 cases with cancer, we found XMRV positivity in 18% of Gleason 6 tumors, 27% of Gleason 7 tumors, 29% of Gleason 8 tumors, and 44% of Gleason 9 tumors (χ^2-test for trend, $\chi^2 = 3.466$, $P = 0.06$, df = 1). Because only 1 case was a Gleason 10, it was not included in the analysis.

Most radical prostatectomy specimens contain relatively low pathological tumor–node–metastasis (TNM) stage cancers, because surgical treatment is not usually performed for higher stages. This is reflected in the distribution of tumor stages (pT) in our series: 75% pT2, 23% pT3, and 2% pT4. XMRV was detected in 25% of stage pT2 tumors and in 32% of pT3 tumors. Of the 5 cases with a pT4 stage, 1 (20%) was XMRV positive (Fig. 4*C*). This moderately increased prevalence of XMRV in advanced stage cancers was not statistically significant. Our sample had very few cases with nodal (N) metastasis and no cases with known distant metastases (M), preventing an investigation of a possible association of XMRV with higher N and M stages. We saw no association between XMRV infection and age at diagnosis (Fig. 4*D*).

XMRV Infection Is Independent of the R462Q Polymorphism of *RNASEL*. XMRV was initially discovered in prostate cancers from men homozygous for a common variant of the antiviral enzyme RNase L. This R462Q amino acid substitution results in a 3-fold reduction of enzymatic activity (8). In their study of 86 men with prostate cancer, Urisman et al. reported that 89% of XMRV-positive cases were homozygous for the R462Q variant (QQ) as compared to 16% of XMRV-negative cases (6). We profiled our 334 cases for the RNase L R462Q variant. The distribution was similar between cases with prostate cancer and controls (42.9% RR, 47.2% RQ, and 9.9% QQ in cancers vs. 52.5% RR, 40.6% RQ, and 6.9% QQ in controls, Fig. 4*E*). There was also no difference in allelic distribution between XMRV PCR-positive (50% RR, 43% RQ, and 7% QQ) and PCR-negative cases (42.7% RR, 47.4% RQ, and 10% QQ; Fig. 4*E*). The 2 XMRV-positive controls had RR alleles. When IHC was used to define XMRV-positive and -negative cases, the relative allelic distributions were also similar. We thus found no association between the presence of XMRV and the RNase L R462Q variant.

Discussion

XMRV is a candidate infectious agent for causing prostate cancer. On the basis of sequence comparison, XMRV was classified as a xenotropic murine gammaretrovirus. We present the first experimental evidence in support of this classification. The morphology of XMRV particles was very similar to MoMLV, a related murine gammaretrovirus. Protein products of the 2 viruses had similar molecular weights, and antisera to most proteins of each virus. The notable exception to this was the SU portion of Env, which determines host specificity and sets xenotropic viruses apart from other related murine viruses. XMRV SU-specific antisera did not cross-react with MoMLV-SU, and the 2 proteins share only a 54% similarity (as opposed to 75–96% similarity for other viral proteins). Our findings thus support the classification of XMRV as a xenotropic murine gammaretrovirus.

We developed 2 sensitive and specific assays for the detection of XMRV in tissues. We used these qPCR and IHC assays to demonstrate the presence of XMRV DNA or proteins in 27% of cases in the largest series of human prostate cancers analyzed thus far. We show that XMRV proteins are expressed almost exclusively in cancerous epithelial cells. Moreover, the presence of XMRV correlated with more aggressive, i.e., higher-grade tumors. These findings provide support for a possible oncogenic effect of XMRV and are crucial for designing studies to investigate mechanisms of transformation.

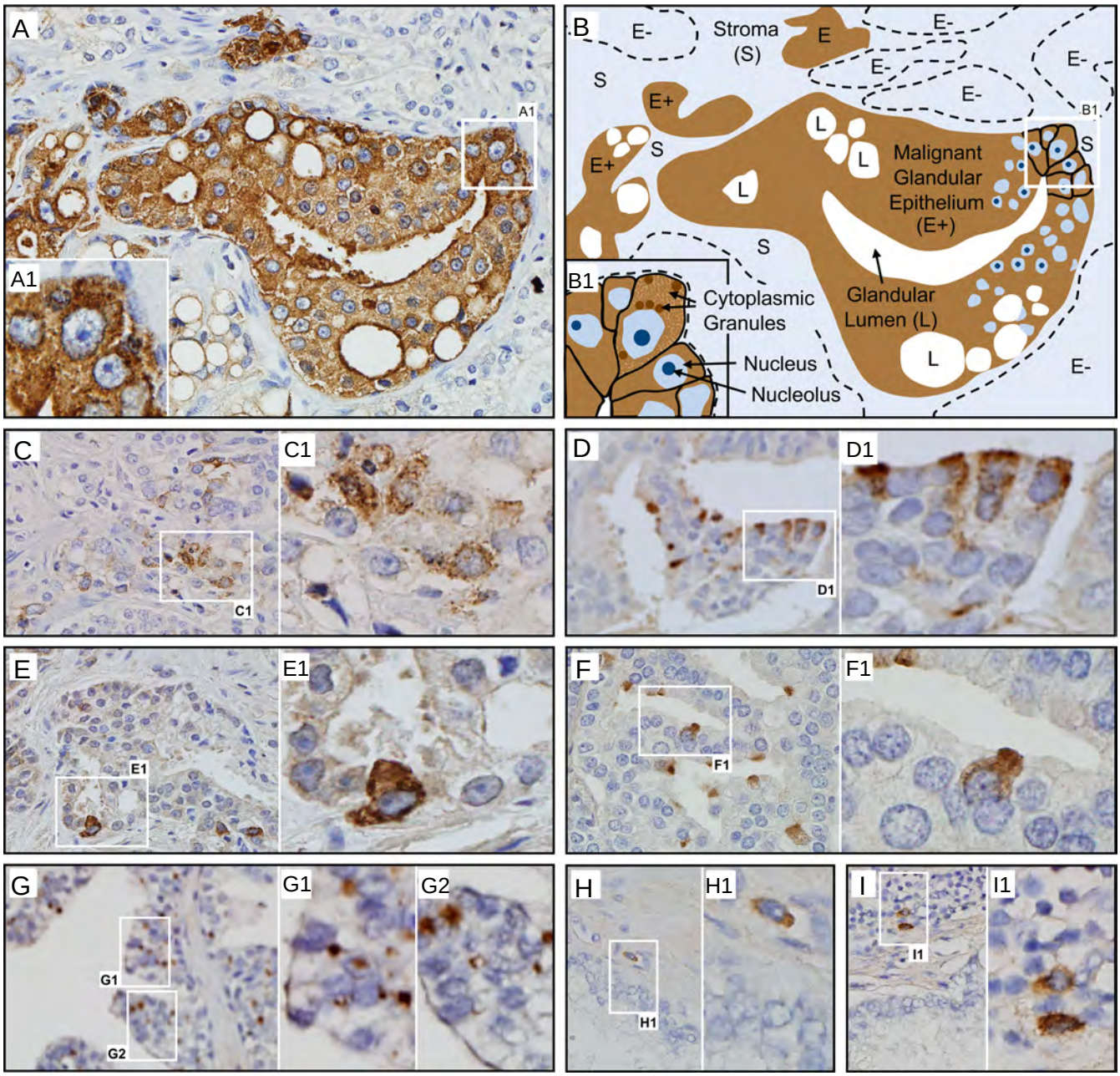

Fig. 3. XMRV proteins are expressed primarily in malignant epithelial cells and very rarely in stromal cells. (*A* and *B*) IHC of a section from a qPCR-positive prostate cancer (*A*) and its diagrammatic representation (*B*). Nuclei of malignant cells are large and contain ≥1 large nucleoli (*B*). Multiple acini of malignant epithelial cells (E+) stain positive. All cells within these acini show intense staining. The stroma (S) and a few other acini (E−) are unstained. *Insets* (*A1* and *B1*) show corresponding fields at higher magnification, with granular cytoplasmic staining pattern in several malignant epithelial cells. (*C*) A different field from the same sample as in *A* shows the range of XMRV protein expression in various acini: fewer cells expressing less protein but the same granular staining pattern. (*D–F*) Three additional representative samples with different frequencies of malignant epithelial cell clusters and different extents of XMRV protein expression. The intracellular staining pattern remains granular in all. (*G*) Staining limited to part of the cytoplasm of malignant epithelial cells in a subset of samples, as in this sample from which the XMRV clone VP62 was isolated, courtesy of R. H. Silverman and C. Magi-Galluzzi, Cleveland Clinic (6). (*H* and *I*) Scattered rare stromal cells showing cytoplasmic staining were seen close to malignant cells (*H*) or within inflammatory infiltrates (*I*).

The fraction of cases positive for XMRV by qPCR (6%) was lower than by IHC (23%). This variation can be attributed to sampling differences in conjunction with very low viral loads. For the qPCR, detection rates depend on the *proportion* of XMRV-infected cells in the tissue. DNA from infected cells gets diluted in DNA from uninfected cells, thus limiting sensitivity if only a few cells in the sample harbor XMRV. However, qPCR allows a rapid survey of large numbers of tissue samples. In contrast, IHC detects individual XMRV-infected cells, avoiding the dilution effect of PCR. However, the number of cells analyzed is much smaller by IHC (a 5-μm section vs. a 100-μm section for DNA extraction) and only actively replicating virus can be detected. Because XMRV produces focal, low-level infections, the 2 assays complement each other and using both is likely to lead to the most accurate estimate of prevalence.

Two of our findings differ significantly from the initial report on XMRV (6). First, we found XMRV proteins in malignant epithelial cells in contrast to initial reports of XMRV proteins in nonmalig-

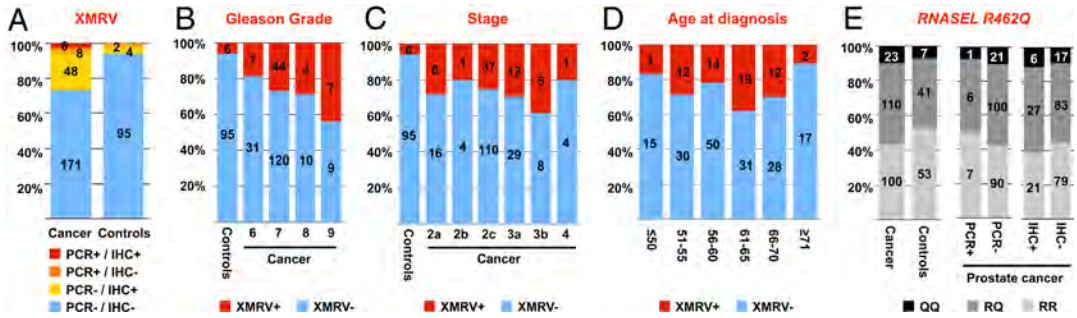

Fig. 4. XMRV DNA and proteins were more prevalent in prostate cancer than in controls, and especially frequent in high-grade cancers, and there was no correlation between presence of XMRV and any particular *RNASEL* genotype. (*A*) Number of prostate cancers or controls that were positive or negative for XMRV, either by qPCR or by IHC. (*B–D*) XMRV-positive cases (by either IHC or qPCR) correlated with Gleason grades (*B*), tumor stage (*C*), or age at diagnosis (*D*). (*E*) Presence of XMRV DNA or protein and the *RNASEL* genotype. Relative frequencies of RR, RQ, and QQ alleles in *RNASEL* at residue 462 were compared in prostate cancer cases and controls (*Left*), in cancers that tested positive or negative for XMRV DNA by qPCR (*Center*), and in cancers that tested positive or negative for XMRV proteins by IHC (*Right*). Cases are shown as percentages of total on the *y* axis and as number of cases within columns.

nant stromal cells. This can be mostly explained by our use of XMRV-specific antiserum instead of the monoclonal antibody to spleen focus-forming virus Gag protein used in the initial report. We were also able to detect XMRV in malignant epithelium from a case in the initial report (Fig. 3*G*), supporting the notion that antisera specific to XMRV offer a more sensitive means of viral detection. Second, we did not see any association of XMRV with the RNase L R462Q polymorphism as described initially. Methodological differences might account for this discrepancy. We tested prostate cancers for the presence of XMRV DNA and protein, whereas Urisman et al. used a nested RT-PCR to amplify viral RNA. It is conceivable that the reduced-activity variant of RNase L has a more significant effect on the levels of XMRV RNA, rather than on infection per se. Given low viral loads, the chance of detecting XMRV RNA may, therefore, be greater in homozygous individuals. Alternatively, the strength of association may depend on allelic frequencies and prevalence of XMRV. The distribution of RNase L R462Q alleles differed significantly between the 2 studies (23% QQ, 16% RQ, 61% RR in the study by Urisman et al. vs. 10% QQ, 43% RR, and 47% RQ in this study). Consistent with our findings, a survey in Northern European patients identified 2 individuals with XMRV; neither was homozygous for R462Q (9). The independence of XMRV infection from the RNase L R462Q variant indicates that all individuals may be at risk for XMRV infection, not just the ≈10% of the population that is homozygous for R462Q. Preventive and antiviral measures will thus benefit a much larger at risk population.

Our finding that XMRV is present in cancerous epithelial cells has important implications for pathogenesis. If XMRV plays a role in prostate cancer development, but infects only nonmalignant stromal cells in the tumor as previously reported, new mechanisms of retroviral oncogenesis would need to be invoked. This finding has discouraged investigation of a causal role of XMRV in prostate cancer thus far. While such a new mechanism is possible, our findings are immediately compatible with classical mechanisms of cell transformation by retroviruses. Retroviruses follow 3 distinct pathways when transforming cells. The first is transduction by an oncogene, where a cell-derived oncogene such as *src* in the viral genome causes rapid transformation. The second is via an essential retrovirus gene transactivating cellular growth-promoting genes, as in the case of the Tax protein of HTLV-I that induces T cell leukemia (10, 11), or the Env protein of Jaagsiekte sheep retrovirus that induces lung cancer in sheep (12). XMRV contains no recognizable oncogene, but we do not understand each XMRV protein enough to rule out any role it might play in transactivation. Finally, there is the insertional activation of a cellular oncogene, a mechanism followed by most leukemia-causing murine gammaretroviruses. Multiple rounds of viral infection are typically needed for the activating insertion to occur. Cells containing the activating insertion are selected over others, leading over time to a distinctly clonal population. While a small number of XMRV integration sites have been sequenced from human prostate cancers (13, 14), no evidence of clonality has emerged yet. Furthermore, the mechanism of insertional activation requires that each cancer cell contains a provirus or, at a minimum, the regulatory sequences from 1 LTR. We estimated that qPCR-positive prostate cancers contained 1–10 copies of XMRV DNA per 660 diploid cells. Because the number of malignant cells in any section varies widely between cases and even between different sections in the same prostate, it is impossible to estimate how many copies of XMRV DNA are present in each *cancer* cell. Our IHC data show that not all malignant cells express XMRV proteins, a finding with 2 possible explanations. It is possible that the malignant cells that lack XMRV protein expression were never infected by XMRV at all—a possibility that is incompatible with any known mechanism of insertional activation by murine gammaretroviruses. Alternatively, it is possible that some XMRV-infected cells lose large portions of their proviral DNA over time, as seen in tumors induced by avian leukosis virus (ALV). In these ALV-induced tumors, an absence of proviral sequences essential for production of viral RNA in most cells, coupled with the absence of viral RNA in tumors, indicates that expression of viral genes is not required for maintenance of the tumor phenotype (15). More studies are required to determine whether XMRV plays any causal role in prostate cancer or whether the presence of the virus in malignant prostatic epithelium is simply a function of its preferential replication in prostate cancer cells.

In line with a slow mechanism for oncogenesis, detection of XMRV in 6% of our controls might indicate that XMRV causes cancer only after a long induction period. Alternatively, these cases may have cancer in an unsampled area of the prostate: TURP removes periurethral tissue whereas cancer usually arises in the periphery of the prostate. It is also possible that XMRV infection does not always lead to cancer. Because our study protocol involves de-identified samples, follow-up of these XMRV-positive controls is not possible.

The finding that XMRV replicates efficiently in a cell line derived from human prostate cancer but not in other human cell lines suggests a viral tropism that warrants further investigation. Is the virus associated with cancers in tissues other than the prostate or in gynecologic malignancies? How is XMRV transmitted? These are all intriguing questions that deserve further exploration. There is growing evidence that current prostate cancer screening algorithms result in early detection of cancers but do not effectively reduce mortality (16, 17). Many cases of prostate cancer are unlikely to manifest themselves during the patient's lifetime. There is a clear need for better markers to detect cancers that pose a significant

health threat and to specifically target these for therapy. XMRV, because of its association with more aggressive cancers, might provide such a marker. Furthermore, there are often cases where a screening test is positive, but no tumor is detected on multiple biopsies, leaving the patient and his physician with no clear guidelines. A second XMRV-specific marker might provide further guidance. Large epidemiologic studies are needed to investigate correlation of XMRV with prostate cancer prognosis. The recognition that human papilloma viruses most often initiate cervical carcinomas has focused efforts on viral detection for early diagnosis and on preventive vaccination. Similarly, a determination that a retrovirus can cause prostate cancer would focus efforts on preventing transmission, antiviral therapy, and vaccine development. The pharmacological inhibition of viral replication, as achieved with HIV-1, could dramatically limit the pathological consequences of chronic viral infection.

Materials and Methods

Creating an Infectious Clone of XMRV. Overlapping partial clones AM-2–9 and AO-H4 derived from patient isolate VP62 (6) (gift of Don Ganem, UCSF) were used to generate pXMRV1, a full-length clone of XMRV with a CMV promoter (details of construction and sequencing are in *SI Text*).

Cell and Virus Production and Assay for Reverse Transcriptase Activity. 293T cells were maintained in DMEM and LNCaP cells in RPMI, both supplemented with 10% FBS, L-glutamine (2.2 mM), penicillin (100 units/mL), and streptomycin (100 μg/mL). Cells were transfected with plasmid pXMRV1 or control plasmid pEGFP-C1 (Clontech), using Lipofectamine PLUS (Invitrogen) following manufacturer's directions. Supernatants were harvested at regular intervals, passed through a 0.45-μm filter (Whatman), and monitored for virus production by measuring RT activity ((18), details in *SI Text*).

Transmission Electron Microscopy (TEM). Virions were centrifuged through 20% (wt/vol) sucrose, resuspended in 2.5% glutaraldehyde in 0.1 M Sorenson's buffer, and processed for TEM as described (19). Samples were analyzed on a JEOL JEM-1200 EXII electron microscope and photographed using an ORCA-HR digital camera (Hamamatsu). Diameters of 100 virions and cores were measured in Adobe Photoshop.

Anti-XMRV Antisera and Western Blot Analysis. For generation of XMRV whole virus antiserum (anti-XMRV), supernatant from cultured, infected cells was passed through a 0.22-μm filter (Pallcorp); centrifuged (18, 20); lysed with detergent and inoculated into rabbits (details in *SI Text*). The rabbits were bled before inoculation for preimmune control sera. Western blot analysis of concentrated virions was performed as previously described for MoMLV (18–20). XMRV proteins were visualized with primary rabbit anti-XMRV, anti-MoMLV CA (NCI 79S-804), anti-MoMLV MA (76S-155), anti-MoMLV NC (80S008, 1:7,500), and anti-XMRV-SU (1:500) antisera (MoMLV antisera and XMRV anti-XMRV-SU antisera were gifts of J. Rodriguez and S. P. Goff, Columbia University, New York). Data from at least 2 independent Western blots were used to determine XMRV protein sizes by comparison against molecular weight markers. MoMLV (NC_001501) was used for sequence comparisons.

Acquisition of Human Prostate Samples: Cancer and Control Tissues. Radical prostatectomy specimens (n = 233) acquired at the Columbia University Medical Center (CUMC) between August 2006 and December 2007 were used to estimate the prevalence of XMRV in human prostate cancer. Prostate tissues removed by TURP for benign prostatic hyperplasia between January 2007 and April 2008 were used as controls (n = 101). Details of tissue acquisition by banks, specimen selection, and processing are described in *SI Text*. Protected health information was removed and samples were de-identified by the tissue bank. Information about age at time of surgery, ethnicity, tumor stage, and tumor grade was retained (Table S1). Experiments were performed in accordance with the Institutional Review Board of CUMC (IRB-AAAC0089).

DNA Extraction from Human Prostate Tissues. DNA was extracted from 10 sections (10-μm thick) of FFPE tissue, quantified (Nanodrop 1000, Thermo Scientific), and stored at −80 °C (details in *SI Text*).

Quantitative PCR Amplification of Proviral DNA. BLAST analysis of overlapping 250-bp segments of the XMRV genome (VP35, GenBank ID DQ241301.1) identified a region of the integrase gene of XMRV that is 100% conserved between VP35, VP42, and VP62 but shares only 80–85% sequence identity with the most similar murine retroviruses. A forward primer, a hydrolysis probe, and 2 reverse primers were selected from this region using PrimerExpress (Applied Biosystems) (details in Table S2 and *SI Text*).

Immunohistochemistry. FFPE cultured XMRV-infected cells and prostate tissues were sectioned at 5-μm thickness and used for IHC. Details of sectioning, antigen retrieval, antibody treatment, counterstaining, protocol optimization, and controls are in *SI Text*.

RNase L Genotyping. The TaqMan SNP genotyping assay (assay ID: C___935391_1_), with the TaqMan SNP Genotyping Mix (both from Applied Biosystems), were used for RNase L G1385A (R462Q) genotyping (NCBI SNP reference: rs486907). Nine nanograms of prostatic DNA was used in a reaction volume of 20 μL. A TaqMan 7500Fast instrument was used for amplification, detection, and allelic discrimination. *RNASEL* genes from 2 individuals of each genotype were sequenced to confirm allelic discrimination results. DNA from 1 individual of each genotype was used as control in each subsequent experiment.

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Xenotropic Murine Leukemia Virus-Related Virus Establishes an Efficient Spreading Infection and Exhibits Enhanced Transcriptional Activity in Prostate Carcinoma Cells▽†

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Xenotropic murine leukemia virus-related virus (XMRV) is a novel human gammaretrovirus discovered in association with human prostate tumors. XMRV was first identified in prostate stromal cells surrounding the tumors of patients carrying a mutation in the *HPC1* gene locus. To determine the tropism of XMRV in cell culture, we tested the ability of XMRV to spread and replicate in various prostate and nonprostate cell lines. We found that although the expression of XMRV viral proteins and the spread of infectious virus were minimal in a variety of cell lines, XMRV displayed robust expression and infection in LNCaP prostate tumor cells. The transcriptional activity of the XMRV long terminal repeat (LTR) was found to be higher than the Moloney murine leukemia virus LTRs in both LNCaP and WPMY-1 (simian virus 40-transformed prostate stromal cells). The U3 promoter of XMRV and a glucocorticoid response element (GRE) within the U3 were required for the transcriptional activity in LNCaP cells. Coexpression of the androgen receptor and stimulation with dihydrotestosterone stimulated XMRV-LTR-dependent transcription in 293T cells, and the GRE was required for this activity. These data suggest that XMRV may replicate more efficiently in LNCaP cells in part due to the transcriptional environment in LNCaP cells.

Nearly 35% of familial prostate cancer patients carry a germ line mutation (R462Q) in the *HPC1* gene locus (15). This locus encodes the protein RNase L, which is expressed and activated upon virus infection and degrades single-stranded viral and cellular RNA, thus blocking replication of the infecting virus and inducing apoptosis (1, 16). The association of prostate cancers with this variant of RNase L raised the possibility that mutant individuals were more susceptible to an unknown tumor virus (2, 15). Total polyadenylated RNA from prostate tumors that were either heterozygous or homozygous for the mutant RNase L allele was isolated and hybridized to a DNA microarray (Virochip) containing oligomers of ~950 viral genomes (19). Seven of eleven tumors that carried at least one allele of the RNase L mutation were positive for the novel retrovirus. Isolation and sequencing of the virus from three different prostate cancer patients revealed nucleotide similarities to xenotropic murine leukemia viruses (MLVs), and the virus was named xenotropic MLV-related virus (XMRV) (19). The genome structure of XMRV is typical of gamma retroviruses. The *env* gene encodes a glycoprotein homologous to the MLV envelope protein that mediates virus binding to the xenotropic receptor, XPR1, on the surface of cells (4). In contrast to more complex retroviruses such as lentiviruses, XMRV does not encode any accessory genes, nor does it encode any host-derived oncogenes (3). Fluorescence *in situ* hybridization and immunohistochemistry revealed that a small number of stromal cells surrounding the tumor, but not tumor cells themselves, were positive for XMRV nucleotide sequences and viral proteins, suggesting that XMRV maintains a low level of infection in these tumors and that direct oncogenesis by XMRV might not play a role in prostate tumorigenesis (19).

Recent studies have demonstrated the affinity of XMRV for prostate cells. XMRV was produced at high titers from ~10 integrated copies within the prostate carcinoma cell line 22Rv1 (11). Another study has confirmed the presence of XMRV-infected cells within the prostate but differs significantly from the original report describing XMRV. XMRV was found in 23% of all prostate cancers without correlation to the RNase L R462Q mutant allele. Significantly, malignant prostate epithelial cells were infected with XMRV at a higher rate compared to stromal cells, thus leaving open the possibility of direct oncogenesis by XMRV (14). Amyloidogenic fragments known as semen-derived enhancer of virus infection (SEVI) from prostatic acid phosphatase increased XMRV infectivity at the level of virus entry. XMRV nucleic acid was also found in prostatic secretions of prostate cancer patients, suggesting a possible mechanism of transmission (9).

XMRV has been shown to be sensitive to the antiviral actions of interferon (IFN) (4), a well-characterized antiviral mechanism against pathogenic infections (13). The DU145 prostate cell line treated with IFN-β prior to XMRV infection was more resistant to a spreading infection than cells without IFN (4). LNCaP prostate cells were permissive for XMRV infection in the presence or absence of IFN and were four times more supportive of virus infection than DU145 cells. The role that RNase L plays in regulating XMRV is still unclear: DU145 cells with a modest small interfering RNA knockdown of RNase L showed slower rather than enhanced replication of XMRV, and there was no change in replication with or without IFN treatment (4). Moreover, it is also unknown what effect

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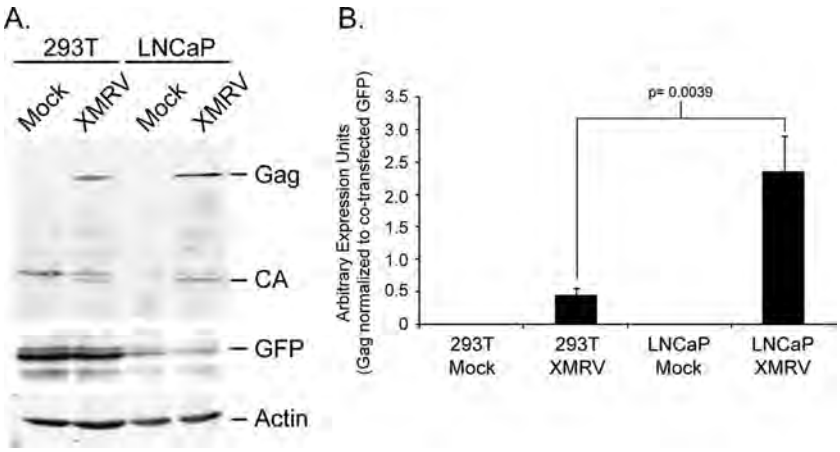

FIG. 1. LNCaP cells express a higher level of Gag and CA compared to 293T cells. (A) Cells were transfected with XMRV DNAs and a plasmid encoding a CMV promoter directing the expression of exogenous GFP. Cell lysates were prepared 2 days posttransfection and characterized for expression of Gag, CA, and GFP levels by SDS-PAGE. (B) Normalization of Gag protein levels to coexpressed exogenous GFP. This experiment was performed in triplicate with the panel A being one representative of the three experiments and panel B showing the quantification of Gag and GFP from all three experiments. *P* values were obtained by using a two-tailed Student *t* test of the data presented.

the R429Q mutation in RNase L plays in the general response against viral infection. The threefold decrease in catalytic activity associated with this mutation may not profoundly change the susceptibility of the cells (2).

In the present study, we determined the ability of infectious XMRV to replicate in cell lines derived from various tissues. Of the cell lines tested, XMRV replicated most efficiently in the LNCaP cell line of prostate origin. To explore why these prostate cells are more permissive for XMRV replication, we analyzed the transcriptional activity of the XMRV long terminal repeat (LTR) in permissive and nonpermissive cell lines. Consistent with the tropism of XMRV replication, an increased transcriptional activity was seen in LNCaP and WPMY-1 prostate cell lines. The U3 region of XMRV and a glucocorticoid response element (GRE) was specifically required for this activity. The data presented here suggest that LNCaP prostate cells provide a transcriptional environment that supports efficient replication and spread of XMRV.

MATERIALS AND METHODS

Cell culture and virus. All cell lines were maintained at 37°C and 5% CO_2 and supplemented with 10% fetal bovine serum (Invitrogen). LNCaP cells (human prostate epithelial tumor cells, a gift of the Gelmann lab, Columbia University) were maintained in RPMI 1640 (Invitrogen). PC-3 cells (human prostate epithelial tumor cells; American Type Culture Collection [ATCC]) were maintained in Eagle modified essential medium (Invitrogen). WMPY-1 cells are prostate stromal cell immortalized with simian virus 40 (SV40) T antigen and were maintained in Dulbecco modified Eagle medium (DMEM). DU145 cells (human prostate carcinoma cells; ATCC) were maintained in FK12 media (Invitrogen). 2fTGH (human fibrosarcoma; gift of Horvath lab, Northwestern University), HeLa (cervical carcinoma; ATCC), TE671 (human rhabdomyosarcoma), Rat2 (rat fibroblast), and 293T (human embryonic kidney) cells were maintained in DMEM (Invitrogen). NIH 3T3 cells (ATCC) were maintained in DMEM supplemented with 10% bovine calf serum (Invitrogen). XMRV virus particles were generated by transfecting LNCaP cells with 5 μg of proviral DNAs. For XMRV spreading infection assays, LNCaP cell culture medium was harvested 8 days postinfection, passed through a 0.45-μm-pore-size filter, and stored at −80°C. Polybrene (8 μg/ml) was added during virus harvesting. The relative concentration of XMRV in supernatants was determined by measuring the reverse transcriptase (RT) activity in the cell culture media of harvested stocks (7). To generate pseudotyped reporter virions, the plasmid Moloney MLV (MoMLV)-XMRV Env, along with pFB-Luc (firefly luciferase with a MoMLV packaging signal), was cotransfected into 293T cells and supernatants were harvested after filtration through a 0.45-μm-pore-size filter.

Plasmids and reagents. The full-length genome of XMRV (patient VP62) was obtained from D. Ganem (University of California, San Francisco). XMRV halves AM 2-9 and AO H4 (19) were joined by introducing a novel MluI site and performing overlapping PCR, which generated one amino acid change: glycine to alanine at position 385 of RT (gift of I. R. Singh, University of Utah). The genome was ligated into the pCR2-TOPO cloning vector. To generate the provirus, the U3 region was amplified and ligated to 5′ R region. This proviral construct was also ligated into the pCR2-TOPO cloning vector and utilized for all subsequent experiments. pcDNA3.1(+) was utilized as a control plasmid for generating mock virus stocks and as a control for mock provirus transfection. MoMLV and XMRV LTR (U3-R-U5-Gag start site) DNAs were amplified by PCR and cloned in between NdeI and HindIII sites by sequence ligation-independent cloning (SLIC) into the plasmid pRL-null (plasmid encoding a promoterless *Renilla* luciferase) and used as a reporter gene. The XM1 reporter plasmid in which the MoMLV U3 was swapped for the XMRV U3 was generated by SLIC cloning in the same manner as the full-length LTRs. The mutant reporter, mGRE, was generated in the same fashion with the nucleotides 192 and 193 (adenine) of the full-length LTRs both being changed to cytosine. The androgen receptor (AR) expression and dihydrotestosterone (DHT) was a gift from Liang-Nian Song of the Gelmann lab (Columbia University [17]). pQXCIP-dsRed (dsRed expression driven by the cytomegalovirus [CMV] promoter) and pCDNA-GFP-PT (green fluorescent protein [GFP] expression driven by the CMV promoter) were utilized as markers for transfection efficiency of 293T or LNCaP cells. pNCA-XMRV Env encodes a full-length MoMLV provirus with the XMRV Env in place of the MoMLV Env. The plasmid pFB-Luc was used as a MoMLV marker for single-round infection assays.

Preparation of cell lysates and immunoblotting. Transfected cells were lysed with NP-40 lysis buffer (150 mM NaCl, 0.5% NP-40, 50 mM Tris [pH 8.0], 0.5 mM EDTA) supplemented with protease inhibitor cocktail (Roche) at 4°C for 30 min. Equal amounts of protein from clarified extracts were added to protein loading buffer, boiled for 5 min, and subjected to SDS-PAGE. Proteins were transferred to nitrocellulose and immunoblotted with antisera against MoMLV CA (goat polyclonal antibody that cross-reacts with XMRV Gag and CA), rabbit polyclonal GFP (ab290; Abcam), or mouse monoclonal β-Actin (A-1978; Sigma) antibody.

XMRV spreading assays. A total of 10^5 Cells were seeded onto six-well dishes and infected the day after with 100 μl of LNCaP culture supernatants containing XMRV viral particles. The cells were allowed to recover after 8 h of adsorption at 37°C with appropriate cell media. Samples were taken each day and subjected to RT assays as described previously (7) to monitor the release of viral particles into the culture supernatants. XMRV was isolated by ultracentrifugation of filter-sterilized (0.45-μm pore size) supernatants at 75,830 × *g* and 4°C for 2 h. Virus pellets were lysed in NP-40 lysis buffer supplemented with protease inhib-

itors and protein loading buffer. Samples were boiled, subjected to SDS-PAGE, and immunoblotted with antisera to the MoMLV CA protein.

Luciferase reporter gene assays. Luciferase reporter gene assays were performed according to the manufacturer's protocol (Promega). 293T, LNCaP, or WPMY-1 cells were transfected with different reporter genes and lysed with passive lysis buffer 24 h posttransfection. For treatment with DHT, cells were exposed to 10 μM DHT at the time of transfection, and all experiments were performed using charcoal-stripped serum. Luciferase activity was measured from triplicate samples using a POLARstar Omega plate reader (BMG Labtech). All conditions represent the average values from triplicate samples, normalized to cotransfected firefly luciferase (pcDNA4.0-Fluc).

RESULTS

To generate infectious virus particles, we obtained the XMRV full-length genome isolated from patient designated VP62 (19). The provirus was transfected into both 293T or LNCaP prostate cells and lysates of transfected cells were tested for XMRV Gag and CA accumulation 2 days posttransfection (Fig. 1). Both 293T and LNCaP lysates contained nearly the same amount of steady-state Gag and CA proteins. The transfection efficiency of LNCaP cells is extremely poor, as indicated by the levels of exogenously expressed GFP. To rule out the possibility that GFP was expressed poorly in these cells, expression vectors encoding either dsRed or GFP were transfected, and fluorescent cells were quantitated by fluorescence-activated cell sorting (FACS) analysis. Although the number of transfected LNCaP cells was less, the fluorescence intensity of dsRed- or GFP-positive cells in both 293T and LNCaP cells were the same, indicating the marker was expressed equally well in transfected cells (data not shown). Normalization of Gag proteins to cotransfected GFP levels suggests that LNCaP cells express a higher amount of Gag compared to 293T cells (Fig. 1B).

To determine whether the XMRV particles are infectious, LNCaP culture media containing XMRV virus particles were applied to 293T or LNCaP cells (Fig. 2A), and these infected cultures were monitored for virus release into the media by analyzing CA accumulation in the supernatants. At 4 and 8 days postinfection, distinctly higher levels of CA at steady state were found in the culture media from infected LNCaP cells compared to 293T cells, suggesting that XMRV spreads more efficiently in LNCaP cells. Virion spread and release into the media were also examined by measuring the activity of RT in the cell culture supernatants (Fig. 2B). LNCaP cells were supportive of an XMRV spreading infection, with peak RT activity detected at day 3, and then continued at day 7, after the cells were reseeded. RT activity in 293T cell culture supernatants, however, was not detected, indicating a lack of replication. Although 293T cells were poor producers of XMRV virus particles, we tested whether the virions from 293T culture media were infectious by applying supernatants to naive LNCaP or 293T cells and monitoring RT activity. Although 293T cells did not support efficient XMRV replication and spread, LNCaP cells rescued the few virus particles produced from 293T cells and could then initiate a spreading XMRV infection (Fig. 2C).

To further assess the cell line tropism of XMRV, we infected seven different cell lines with XMRV harvested from LNCaP cell culture supernatants. XMRV infection was conducted on LNCaP, 293T, HeLa (human cervical carcinoma), 2fTGH (human fibrosarcoma), TE671 (human rhabdomyosarcoma), and Rat2 (rat fibroblast) cells, and subsequent CA release into the culture media was examined (Fig. 3A). Three days postinfection, CA could be detected in the media supernatants from all cell lines, but LNCaP supernatants contained the highest levels. After 6 days, high levels of CA were observed in culture supernatants from LNCaP cells but not from the other cell lines, indicating that XMRV infection and spread was most efficient in LNCaP prostate cells. Next, we tested XMRV replication in three other prostate cell lines: DU145 prostate carcinoma cells, WPMY-1 prostate stromal cells immortalized with T-antigen, and PC-3 prostate carcinoma cells. As before, XMRV supernatants were applied to naive cells and release of

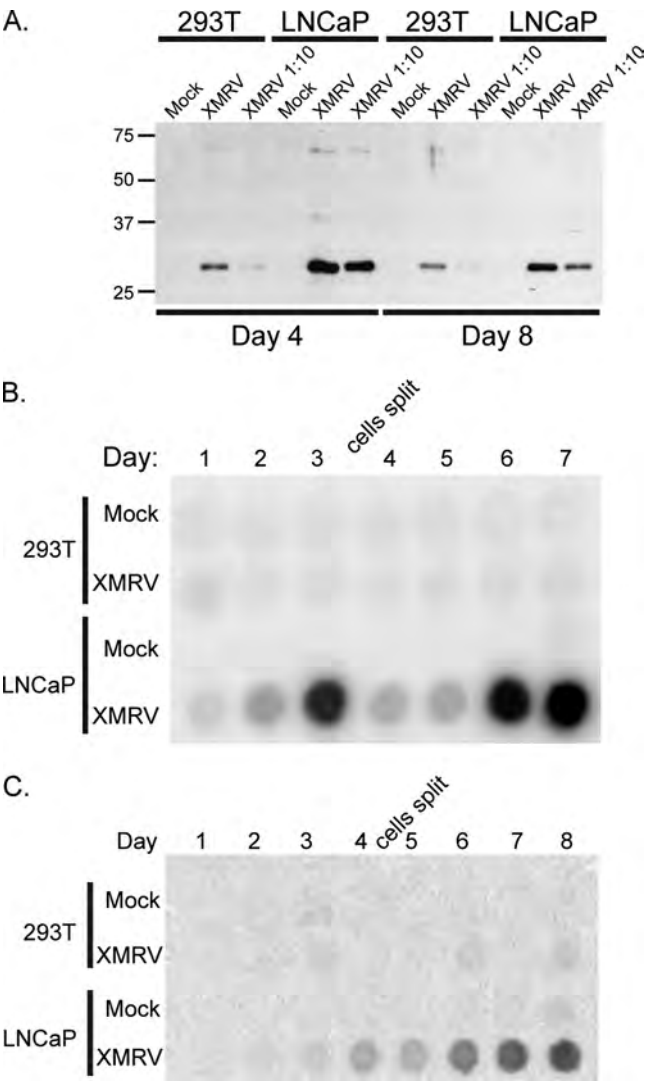

FIG. 2. XMRV spreads efficiently in LNCaP cells but not 293T cells. (A) Eight-day culture media from LNCaP cells that were transfected with the XMRV provirus were adsorbed onto either naive 293T or LNCaP cells. Virus spreading was monitored over 8 days by immunoblotting supernatants against XMRV CA. XMRV 1:10 culture medium was diluted 10-fold at the time of infection. (B) Same as in panel A, but with monitoring of RT activity over 7 days. On the third day, the cells were diluted and reseeded to allow accumulation of XMRV virus particles. (C) Same as in panel A, but culture media from 293T cells that were transfected with the XMRV provirus were adsorbed onto either naive 293T or LNCaP cells. The results shown are representative of five different experiments.

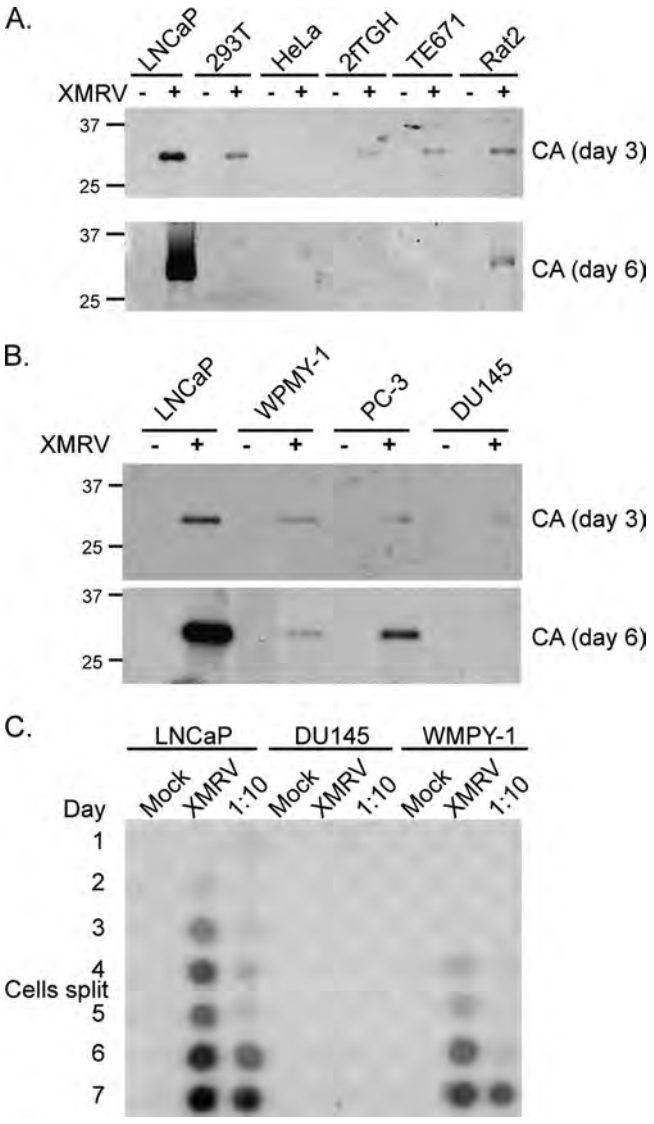

FIG. 3. XMRV spreading in LNCaP cells. (A) Same as in Fig. 2, but different cell lines were infected with XMRV and levels of CA were measured by immunoblotting at 3 and 6 days postinfection. 293T, human embryonic kidney cells; HeLa, human cervical carcinoma; 2fTGH, human fibrosarcoma; TE671, human rhabdomyosarcoma; Rat2, rat fibroblast. (B) Same as in panel A, but three prostate cell lines were tested: WPMY-1 (prostate stromal immortalized with SV40 T-antigen), DU145 (epithelial prostate carcinoma), and PC-3 (prostate epithelial). (C) RT assay measuring XRMV spread in three different prostate cell lines: LNCaP, DU145, and WPMY-1 cells. The results shown are representative of three different experiments.

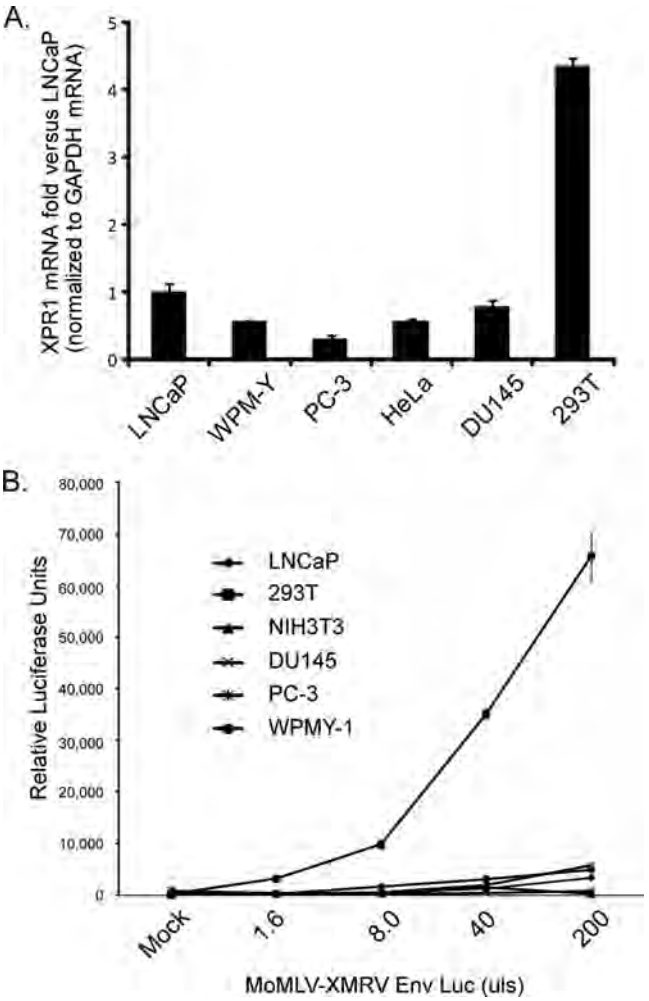

FIG. 4. 293T cells mediate a higher amount of viral entry at the level of receptor. (A) Total mRNA isolated from LNCaP, WPMY-1, PC-3, HeLa, DU145, 293T, and 2fTGH cells were reverse transcribed to obtain cDNA. Triplicate RT products were amplified by using primers for XPR1 or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by quantitative PCR and SYBR green reaction mix. Standard curves for both XPR1 and GAPDH were created and XPR1 values were normalized to GAPDH transcript levels. The data are represented as the fold difference against LNCaP XPR1 mRNA levels where LNCaP XPR1 = 1. (B) A total of 2.5×10^5 cells of each cell type were infected with MoMLV virions that contain the XMRV Env and a packaged firefly luciferase reporter (MoMLV-XMRV Env Luc). To obtain data from a single round of infection, lysates were prepared 24 h postinfection and analyzed for luciferase activity. Each sample was normalized to total protein levels, and all analyses were performed in triplicate.

CA in the culture media was measured (Fig. 3B). Compared to other prostate cell lines, LNCaP cells again were the most permissive for XMRV replication and spread, as monitored by CA production. RT release into the media was detected in LNCaP, DU145, and WPMY-1 cells (Fig. 3C), with LNCaP cells supporting the most robust spreading infection.

Next, we determined whether XMRV replicates efficiently in LNCaP cells due to increased viral entry at the level of the cellular receptor, XPR1. Analysis of XPR1 endogenous mRNA levels in different cell lines (Fig. 4A) demonstrated that whereas all of the cell lines tested expressed relatively small amount of XPR1 transcripts (data not shown), 293T cells had the highest XPR1 transcript levels. MoMLV particles that were psuedotyped with XMRV Env and contained a packaged luciferase reporter gene was used to infect cells and analyze virus entry in a single round of infection (Fig. 4B). Interestingly, although most cell lines, including LNCaP cells, exhibited low luciferase activities, 293T cells, which are largely refractory to XMRV spread, supported a robust degree of virus entry. These data suggests the amount of expressed receptor

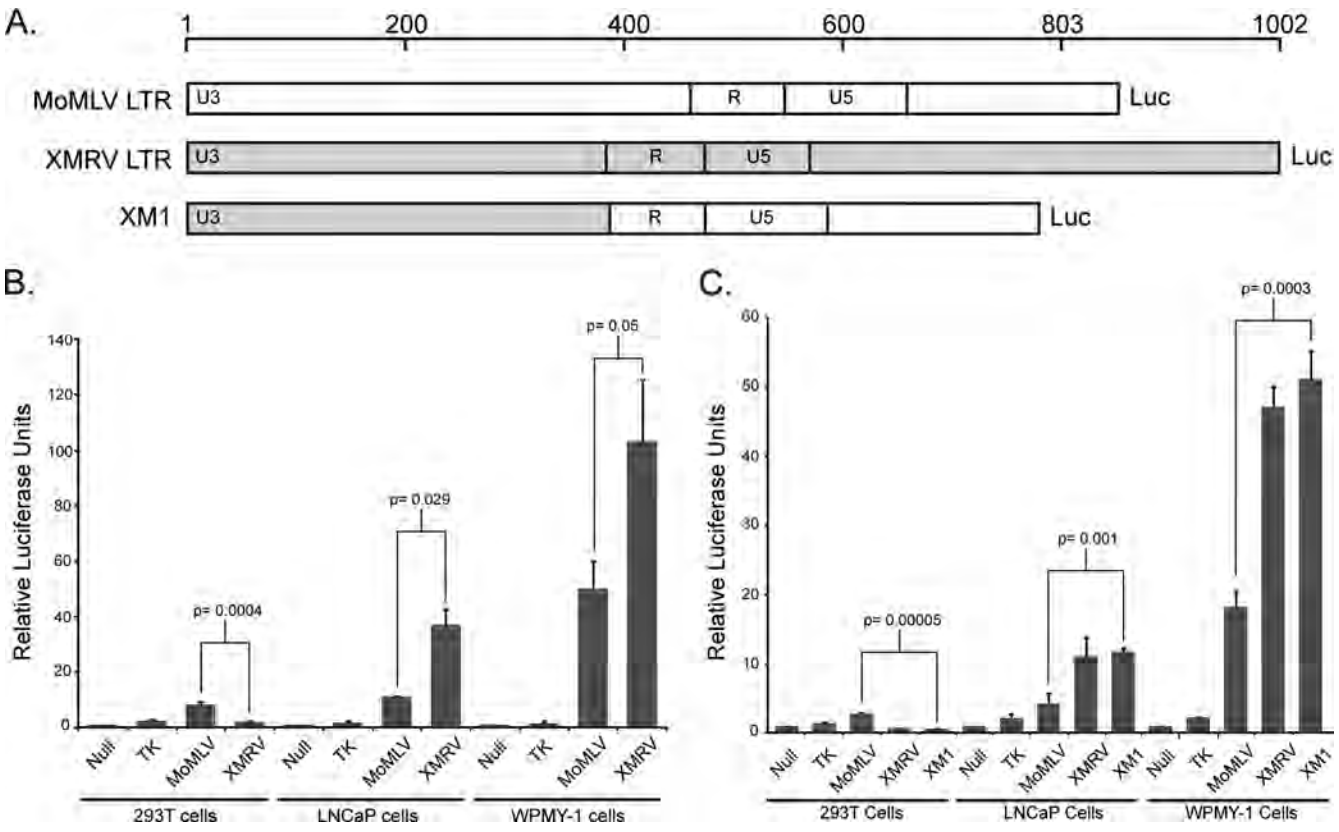

FIG. 5. XMRV LTR exhibits a higher transcriptional activity in LNCaP and WPMY-1 prostate cells. (A) Schematic diagram of MoMLV and XMRV LTR luciferase reporter gene constructs. The 5′ LTR of both MoMLV and XMRV were fused to *Renilla* luciferase at the translational start site for Gag. XM1, chimeric reporter gene where the U3 region of MoMLV was swapped for the U3 of XMRV. (B) Reporter genes in panel A were transfected into 293T, LNCaP, or WPMY-1 cells, and the luciferase activity was measured 24 h later. Null, luciferase with no promoter; TK, HSV-1 thymidine kinase promoter upstream of *Renilla* luciferase. All samples were assayed in triplicate and normalized to cotransfected firefly luciferase. The data are represented as the fold difference compared to the null control (null = 1). (C) Same as in panel B, but the XM1 reporter gene was included, demonstrating that the XMRV U3 is required for the observed transcriptional specificity in LNCaP and WMPY-1 cells. *P* values were obtained by using a two-tailed Student *t* test of the data presented. This experiment was repeated three times.

and viral entry is not a determining factor in the spread of XMRV in LNCaP cells.

The intracellular accumulation of XMRV Gag and CA to higher levels in LNCaP cells suggested the possibility that the promoter of XMRV displayed an enhanced level of transcription in these cells. We generated luciferase reporters that were fused to the LTRs of both MoMLV and XMRV, in which the fusion point was the translation initiation site of Gag (Fig. 4A). We then tested the transcriptional output of these reporter genes by transfecting LNCaP, WPMY-1, or 293T cells with these DNAs and measuring luciferase activity (Fig. 4B). The transcriptional activity of the MoMLV LTR was higher than the herpes simplex virus type 1 (HSV-1) TK promoter in all cell lines tested, but the pattern of XMRV LTR activity in different cells was distinct. The XMRV LTR transcriptional activity was lower than that of MoMLV in 293T cells but higher in both LNCaP and WPMY-1 prostate cells.

Most, if not all, of the transcriptional activity of the retroviral LTR typically originates from *cis*-acting DNA elements within the U3 region that recruit various cellular transcription factors. Whether the XMRV U3 was responsible for the enhancement of XMRV transcription in LNCaP and WPMY-1 cells was tested by generating a chimeric luciferase reporter gene in which the MoMLV U3 was replaced with the U3 of XMRV (Fig. 5A) and tested for its transcriptional activity (Fig. 5C). The chimeric reporter gene (XM1) behaved exactly like the full-length XMRV LTR: transcriptional activity was lower than MoMLV LTR in 293T cells but higher in LNCaP and WMPY-1 prostate cells. Together, these data indicate that the U3 region of the XMRV LTR promotes transcription more efficiently in LNCaP and WMPY-1 prostate cell lines.

Xenotropic MLVs contain a GRE within their U3 region which is conserved within the XMRV LTR. To determine whether the XMRV GRE binding site plays a role in transcription, the GRE was mutated (GAACAGATGG to G**CC**CAG ATGG; mGRE), and the promoter activity of the mutant LTR (mGRE) was determined by luciferase assays (Fig. 6). Significantly, the mutant mGRE reporter exhibited a fourfold reduction in LNCaP cells, suggesting that this DNA element may play a role in transcription from the XMRV LTR (Fig. 6A). We next tested whether the AR can activate the XMRV-LTR reporter gene in 293T cells (Fig. 6B). Coexpression of AR *per se* had no effect on the transcriptional activity of the XMRV LTR; however, after coexpression of AR and stimulation with DHT, an AR agonist, the activity increased to that of the MoMLV LTR. DHT and AR coexpression had no effect on

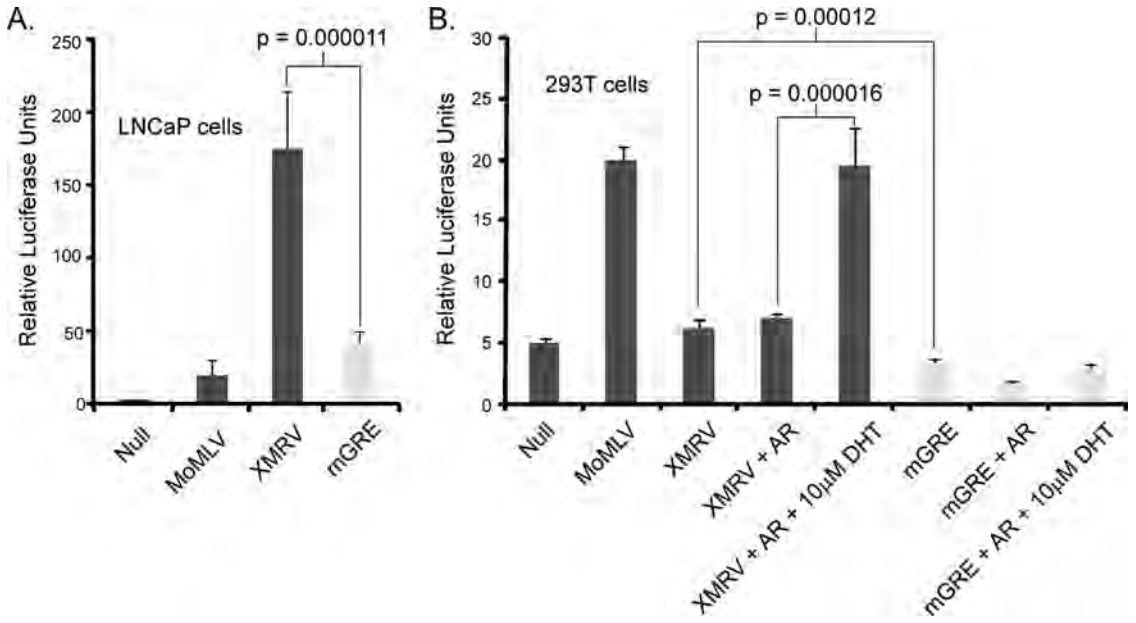

FIG. 6. The GRE site within the XMRV LTR is required for transcriptional activity. (A) 293T cells were transfected with the LTR reporter genes depicted in Fig. 4A and with a XMRV LTR containing a mutant within the GRE site (mGRE; GAACAGATGG to G**CC**CAGATGG). Luciferase activity was measured 24 h later and normalized to cotransfected firefly luciferase. All samples were assayed in triplicate and were normalized to cotransfected firefly luciferase. (B) Same as in panel A, but LNCaP cells were transfected. (C) Same as in panel A, but the XMRV LTR was cotransfected with the AR and treated with DHT for 24 h after lysis. mGRE was included to demonstrate that the mutation does not respond to AR expression and DHT stimulation. *P* values were obtained through a two-tailed Student *t* test of the data presented. This experiment was repeated twice.

the transcriptional activity of the MoMLV reporter gene (data not shown). Importantly, mutation of the GRE site abolished activity with or without DHT stimulation and AR coexpression. Coexpression with glucocorticoid receptor (GR) and treatment with dexamethasone, a GR agonist, had no effect on the XMRV reporter gene (data not shown).

DISCUSSION

This study examines the tropism of XMRV in different culture cell lines. We found that expression of Gag and CA from the provirus of XMRV is more efficient in LNCaP cells than in 293T cells. Similarly, LNCaP cells supported XMRV viral spread, whereas 293T cells did not. The few virus particles that did arise from 293T cells could be rescued by transfer to LNCaP cells; thus, virions from 293T cells were not grossly defective. Of the prostate and non-prostate cell lines tested, LNCaP cells are the most efficient host cell line for spread of infectious XMRV. We analyzed the transcriptional output from the XMRV LTR and found that the U3 region provides a higher transcriptional activity than the constitutively highly active MoMLV LTR in LNCaP and WPMY-1 cells but lower activity than the MoMLV LTR in 293T cells. These data suggest that the U3 promoter region of the XMRV LTR plays a significant role in transcriptional activity in LNCaP cells.

The U3 regions of xenotropic MLVs are conserved and contained transcriptional elements that regulate transcription of the integrated provirus (18, 20). Transcription factors such as NF-1, E-box proteins, and C/EBP coordinate with other factors to activate transcription signals to RNA Polymerase II. Interestingly, the mouse xenotropic MLVs contain two GREs that are conserved in XMRV. Cells that constitutively express the AR, such as hormone-responsive prostate cells and LNCaP cells, are thus predicted to be susceptible to AR-dependent agonists and could stimulate transcription of XMRV. In support of this notion, coexpression of the AR along with the XMRV LTR reporter in 293T cells, and stimulation with DHT, increased transcription. Mutation of one of the conserved GRE sequences abolished the transcriptional activity observed with DHT stimulation, suggesting that AR and steroid responses do indeed play a role in XMRV replication. Characterization of endogenous AR expression reveals that LNCaP cells express the AR, whereas DU145 and PC-3 cells do not (data not shown), suggesting that the reduced replication in these cell lines may be due to the absence of AR. Indeed, a study by Dong and Silverman (5) demonstrated that stimulating LNCaP cells with DHT increased the transcriptional activity of an XMRV reporter gene, whereas the AR antagonists Casodex and Flutinamide blocked this activity.

Alternative explanations for the enhanced replication of XMRV in LNCaP cells also exist. It is possible, for example, that the cellular receptor for XMRV, XPR1, may be expressed at a higher level in LNCaP cells than other cell types tested in the present study. However, quantitative PCR analysis revealed that 293T cells express fourfold-higher levels of XPR1 mRNA transcripts than LNCaP cells, suggesting that XPR1 does not account for the weak spreading infection of XMRV in 293T cells despite an increase in viral entry. We also considered the possibility that the lack of RNase L expression in LNCaP cells may be responsible for the permissiveness. To address this possibility, we depleted RNase L by using RNA interference in 293T cells. Despite

>95% reduction in RNase L protein levels, XMRV replication was not enhanced (data not shown). However, it remains possible that production of IFN may limit the spread of XMRV. Increasing amounts of IFN exposure in an IFN signaling-competent cell line reduced XMRV replication in a dose-dependent manner (4). LNCaP cells are known to be deficient in JAK1 and have impaired IFN signaling, which may account for the robust spreading in this cell line (6). Experiments in which IFN antiviral signaling is restored in LNCaP cells will ultimately resolve this possibility. Another possibility might be that the lack of expression of an XMRV-restrictive factor in LNCaP cells provides a boost to viral replication. However, we consider this unlikely since the expression of APOBEC3G and Tetherin, both potent inhibitors of retrovirus replication (8, 10), are poorly expressed in 293T cells, and this suggests some other factor may play a role in XMRV spread. Importantly, XMRV was also found in the plasma (peripheral blood mononuclear cells, B cells, and T cells) in a high percentage (67%) of patients with chronic fatigue syndrome, suggesting that XMRV has a broader host range than previously appreciated (12).

We show here that XMRV can replicate efficiently in LNCaP prostate cells and induce the release of high levels of virus. This may in part be due to an enhanced transcriptional environment within LNCaP cells that allows for production of more viral proteins and subsequent budding of viral particles. The increase in transcriptional activity of the XMRV LTR is totally attributable to the U3 region and requires a GRE sequence element within the U3. The data presented further indicate that XMRV transcription can be enhanced by steroids, suggesting that XMRV may show selectivity for hormone responsive cell types, including the prostate.

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